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Looking at plant innate immunity in Genomic Era*

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PREAMBLE

Prof. S. N. Banerjee, the renowned Indian Mycologist attached to the University of Calcutta, being one of the founder members of Indian Mycological Society served the Society as an Editor since its inception in 1954. Later in 1970 he became the President of the Society and served till 1977. He and his associates contributed substantially to the knowledge of higher fungi, especially timber rotting Basidiomycetes. I felt greatly honoured when I have been requested to deliver Prof. S. N. Banerjee 14th Memorial Lecture in the University of Calcutta on 11th January, 2013. As a researcher in the area of Plant pathogen interaction we pay our homage to Prof. Banerjee and his colleagues who devoted to advance the research on physiological and pathological aspects of fungal, microbial organisms. What we described in the present deliberation, the investigation of plant defense mechanism against various pathogens is relied on the strong foundation the stalwarts set about sixty years back.

Crop yield is greatly affected by plant diseases caused by diverse types of organisms. The best way to control plant disease so far has been the cultivation of disease resistant plants. However resistant breeding is limited by non-availability of either or both diverse resources for resistance trait and resistance trait linked molecular markers. Hence research efforts have been diverted to identify sources for novel resistant traits as well as to understand the underlying mechanism of plant immunity. A proper understanding about the machinery adapted by plants to survive against the pathogen is essential for identifying key players in plant defense response. Advances in omi technologies have lead to generation of enormous amount of data and has vastly progressed our knowledge about plant immune responses and signaling pathways. Present review summarizes our present understanding about plant innate immunity and the role played by molecular and omic techniques.

INTRODUCTION

Human civilization is largely dependent on plant and plant products for food security and economy.

Hence, plant diseases have been a matter of major concern and of active research. The magnitude and nature of losses caused by plant diseases vary with the host plant, pathogen, environmental conditions and management practices. The crop loss may be minor to severe depending on combination of these factors. The crop yield is affected in the field as in case of most diseases, or during

storage as in case of rots of fruits and vegetables. Sometimes the losses may be in terms of quality rather than quantity, as in case of spots, blemishes and blotches on fruits or vegetables, thus reducing the market value of the product. The best approach to control plant disease so far has been the cultivation of disease resistant plants. However resistant breeding is limited by non-availability of either or both diverse resources for resistance trait and resistance trait linked molecular markers. Hence research efforts have been diverted to identify sources for novel resistant traits as well as to understand the underlying mechanism of plant immunity. Modern molecular techniques, comparative and functional genomics have not only largely augmented knowledge about the pathways and mechanisms involved in plant defense but also provided us with tools to apply this knowledge for agricultural benefits.

Plant Innate Immunity – development of the concept

The study of plant- pathogen interaction gained a new dimension when H. H. Flor proposed the classical gene-for-gene hypothesis (Flor 1946). He showed that the inheritance of both resistance in the host and pathogenic ability of the disease causing organism is controlled by pairs of matching genes. He proposed that for each gene of resistance (R-gene) in the host there is a corresponding avirulence (avr-gene) in the pathogen and for each gene for virulence in the pathogen these is a gene for susceptibility in the host. In early 1970s, it was first discovered that plants are able to perceive microbe derived compounds resulting in induction of phytoalexin (Anderson-Prouty and Albersheim 1975, Keen 1975). Later, it was discovered that plant cell wall derived polysaccharide may also induce phytoalexin production (Hahn et al. 1981). Further studies showed that plants treated with microbial elicitors become resistant to subsequent pathogen infections (Ayers et al. 1976, Hadwiger and Beckman 1980). These observations gave rise to the concept of plant innate immunity and lead to intense search for identifying more elicitors. Through innovative biochemical and cell cultural techniques many elicitors i.e. oligosaccharides, peptides, lipids as well as plant responses to the elicitors i.e. reactive oxygen species (ROS) production, medium alkalization, protein phospho-

rylation were also discovered (Boller 1995, Felix et al. 1991, Hahlbrock et al. 1995, Kuchitsu et al. 1993). However the receptors in plant cell responsible for perceiving these elicitors remained elusive. It was not until advent of advanced genetic technology in 1990s that the first receptors were isolated from multiple plant species. These included NBS-LRR domain containing protein RPS2 from Arabidopsis (Mindrinos et al. 1994), TIR domain containing Flax L6 (Lawrence et al. 1995) and Tobacco N protein (Whitham et al. 1994), tomato PTO kinase (Martin et al. 1993), rice XA21 receptor kinase (Ikeda et al. 1990, Khush et al. 1990) and tomato receptor-like protein CF9 (De Wit et al. 1985). The discovery of these R-genes and their role in pathogen perception established that plant immunity involves diverse mechanisms and molecules.

Even though many R-genes were identified and predicted to recognize microbial elicitors, based on structural similarity to animal receptors, their interaction with elicitors could not be established as the cognate elicitors remained elusive. A clear idea about receptor-elicitor interaction was obtained with identification of flg22, a peptide present at the N-terminal region of flagellin protein, as a strong elicitor of immune response in Arabidopsis (Felix et al. 1999) and isolation of the corresponding receptor , FLS2 (Gómez-Gómez and Boller 2000).The demonstration that FLS2 binds flg22 provide the first molecular evidence of direct receptor-elicitor interaction (Chinchilla et al. 2006, Zipfel et al. 2004). Further it was discovered that mutation in FLS2 compromised immune response and made Arabidopis susceptible to Pseudomonas syringae. These discoveries ignited the interest of molecular biologists to delineate the mechanism of elicitation of plant immune response; availability to genomic information and techniques of comparative genomics facilitated the discovery of many more R-genes and microbial elicitors (Table 1).

Plant Immune System – an overview

Knowledge so far obtained about molecular structure of elicitors and their cognate receptors is far from complete; nevertheless it provides a framework for understanding plant immune response (Boller and Felix 2009, Jones and Dangl 2006, Nurnberger et al. 2004). Plants have evolved two

Table 1 : Table of confirmed and predicted plant receptors.

Name	Plant	Overall Structure	Ligandbinding domain	Class	Ligand/epitope	Pathogen	Function of ligand
Experimentally Confirmed Receptors							
XA21	Rice	Non-RD RK	LRR	Receptor Kinase	Ax21/AxYs22	*Xanthomonas oryzae pv. oryzae* (Xoo)	Quorum sensing
FLS2	*Arabidopsis*	Non-RD RK	LRR	Receptor Kinase	Flagellin/flg22	*Pseudomonas tabaci*	Mobility
EFR	*Arabidopsis*	Non-RD RK	LRR	Receptor Kinase	Elongation factor/elf18	*Escherichia coli*	Protein translation
EIX	Tomato	RLP	LRR	Receptor-like protein	EIX	Yeast	Cell-wall degrading enzyme
WAK1	*Arabidopsis*	RD RK	EGF-Like	Receptor Kinase	OG	Lytic fragments of plant cell wall	Plant cell wall
CEBiP	Rice	RLP/GPI-anchored membrane protein	LysM	Receptor-like protein	Chitin	Fungi	Cell wall component
LYM1 and LYM3	Arabidopsis	RLP/GPI-anchored membrane protein	LysM	Receptor-like protein	PGN	Bacteria	Cell wall component
Predicted Receptors							
XA26	Rice	Non-RD RK	LRR	Receptor Kinase	Unknown	*Xanthomonas oryzae pv. oryzae* (Xoo)	Unknown
CF-4	Tomato	RLP	LRR	Receptor-like protein	Avr4	*Cladosporium fulvum*	Chitin binding, protection from plant chitinases
SNC2	*Arabidopsis*	RLP	LRR	Receptor-like protein	Unknown	*Pseudomonas syringae pv. tomato*	Unknown

contd. table 1

RLP30	Arabidopsis	RLP	LRR	Receptor-like protein	Unknown	*Pseudomonas syringae* pv. *phaseolicola*	Unknown
RLP52	Arabidopsis	RLP	LRR	Receptor-like protein	Unknown	*Erysiphe cichoracearum*	Unknown
VE1	Tomato	RLP	LRR	Receptor-like protein	Unknown	*Verticillium dahliae* race 1	Unknown
XA21D	Rice	Soluble predicted plasma membrane localized protein	LRR	Extracellular Soluble Receptors	Ax21/AxYs22	*Xanthomonas oryzae* pv. *oryzae* (*Xoo*)	Quorum sensing
Pi-D2	Rice	Non-RD RK	Lectin	Receptor Kinase	Unknown	*Magnaporthe grisea*	Unknown
RLK1	Tobacco	Non-RD RK	Lectin	Receptor Kinase	Potentially CAP-Pa28	*Phytophthora capsici*	Unknown
MBL1	Rice	Soluble predicted plasma membrane localized protein	Lectin	Extracellular Soluble Receptors	D-Mannose containing molecules	*Xanthomonas campestris* pv. *vesicatoria*	Ptential cell wall component
SNC4	Arabidopsis	Non-RD RK	GDPD	Receptor Kinase	Unknown	Unknown	Unknown
CERK1	Arabidopsis	RD RK	LysM	Receptor Kinase	Chitin, PGN	*Pseudomonas syringae* pv. *tomato*	Unknown
GBP	Soybean	Soluble predicted plasma membrane localized protein	Glucan binding	Extracellular Soluble Receptors	Beta-glucan heptamer	*Phytopthora sojae*	Cell wall component
RPG1	Barley	Cytoplasmic plasma membrane localized non-RD kinase	Pseudokinase	Cytoplasmic receptors	Unknown	*Puccinia graminis*	Unknown
RPG5	Barley	Cytoplasmic non-RD kinase	NLR	Cytoplasmic receptors	Unknown	*Puccinia graminis*	Unknown
WKS1	Wheat	Cytoplasmic non-RD kinase	START	Cytoplasmic receptors	Unknown	*Puccinia striiformis*	Unknown

Abbreviations: RD- Arginine-aspartate; RK- Receptor kinase; LRR- Leucine-rich repeat; EIX- Ethylene-inducing xylanase; OG- Oligogalacturonide; EGF- Epidermal growth factor; RLP- Receptor like protein; GPI- Glycophosphatidylinositol; PGN- Peptidoglucan; GDPD- Glycerophosphoryldiester phosphodiesterase; NLR- Nucleotide-binding site; START- StAR-related lipid transfer.
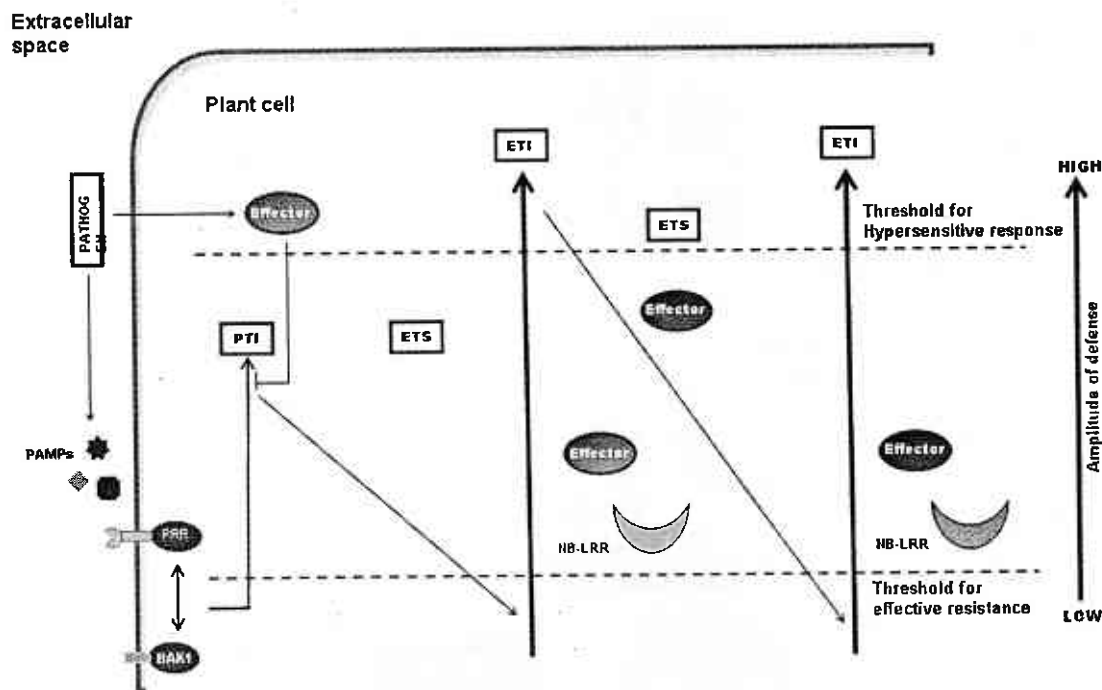
Fig. 1 : PAMPs from plant pathogens are recognized by cell surface PRRs and elicit PAMP-triggered immunity (PTI). Many PRRs interact with the related protein i.e. BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE 1 (BAK1) to initiate the PTI signaling pathway. Pathogens deliver effectors proteins into the host cell, these intracellular effectors often act to suppress PTI. However, many effectors are recognized by intracellular nucleotide-binding (NB)-LRR receptors, which induces effector-triggered immunity (ETI) (Adapted from Jones et al 2006 and Dodds et al 2010).
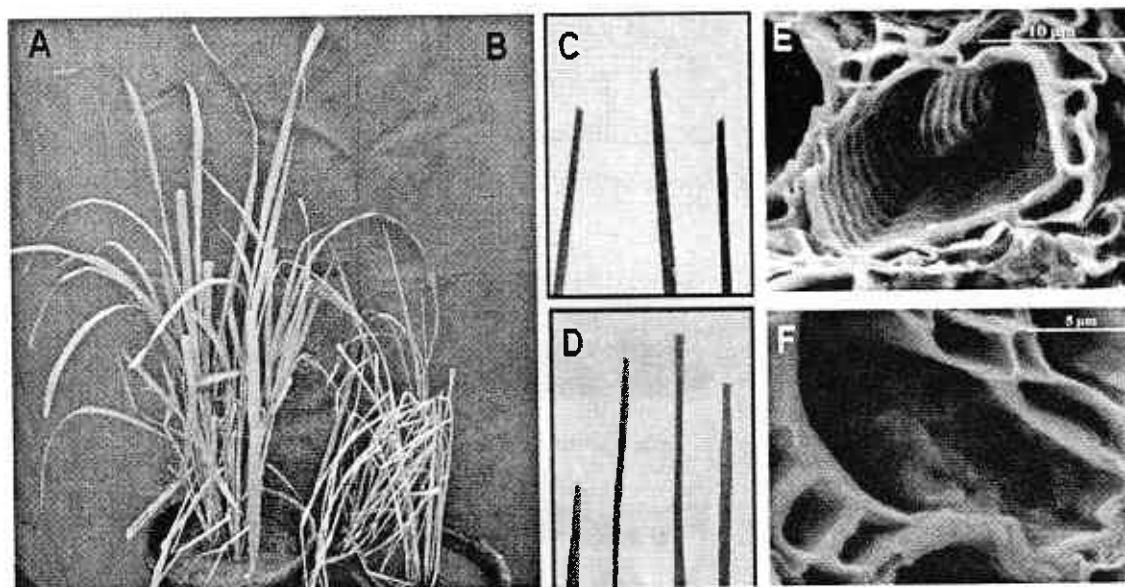

Fig. 2: Symptoms of Bacterial leaf blight disease in Rice caused by *Xanthomonas oryzae* pv. *oryxae* (Xoo) in resistant (IET8585) and susceptible (IR24) plants. A. Xoo inoculated 55 days old resistant plant. B. Xoo inoculated 55 days old susceptible plant. C. Leaves from 18 days old Xoo inoculated resistant plant. D. Leaves from 18 days old Xoo inoculated susceptible plant. E. Scanning Electron micrographs of transverse section of leaves from 18 days old Xoo inoculated resistant plant. F. Scanning Electron micrographs of transverse section of leaves from 18 days old Xoo inoculated susceptible plant.

strategies for detecting pathogens: one uses transmembrane pattern recognition receptors (PRRs), on the external face of the host cells; and the second uses intracellular receptors to detect pathogen virulence effectors (Fig 1) (Ausubel 2005, Chisholm et al. 2006). PRRs recognize the slowly
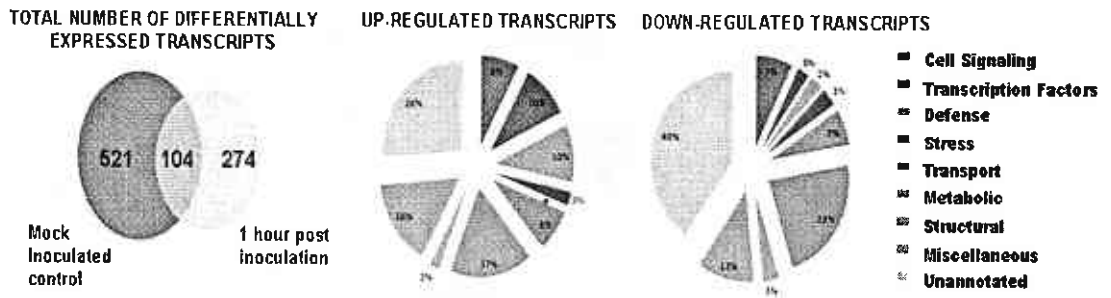
TOTAL NUMBER OF DIFFERENTIALLY
EXPRESSED TRANSCRIPTS
UP-REGULATED TRANSCRIPTS   DOWN-REGULATED TRANSCRIPTS

- Cell Signaling
- Transcription Factors
- Defense
- Stress
- Transport
- Metabolic
- Structural
- Miscellaneous
- Unannotated

521   104   274

Mock   1 hour post
Inoculated   inoculation
control

Fig. 3: Vein diagram representing overlap of total transcripts between mock treated and pathogen treated samples as well as functional categorization of up-regulated transcripts and down-regulated transcripts. (Adapted from Grewal *et al.* 2012).
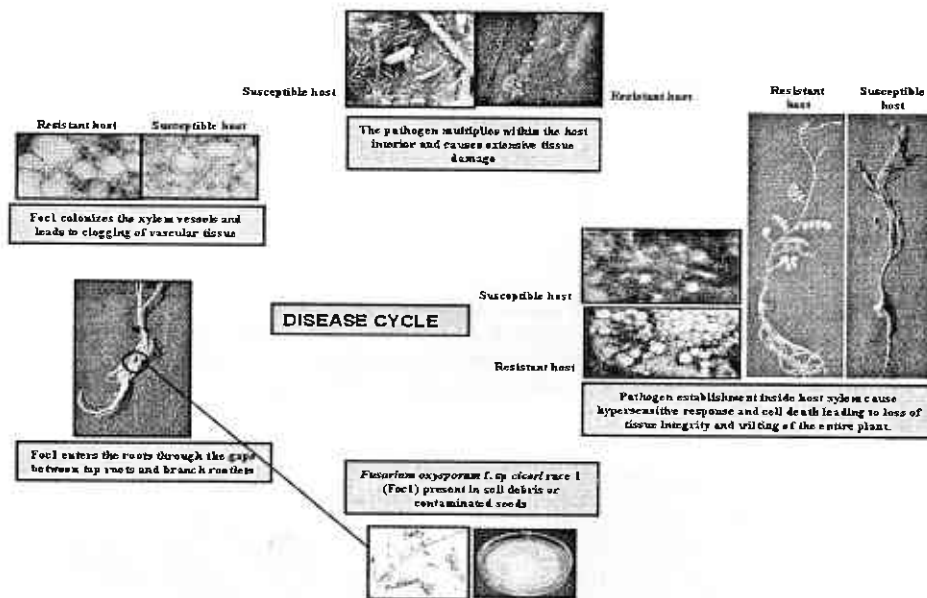

Fig. 4: Schematic diagram showing the disease cycle of *Fusarium oxysporum* f.sp *ciceri* race 1 and *in planta* pathogen progression (Adapted from Gupta *et al.* 2009).
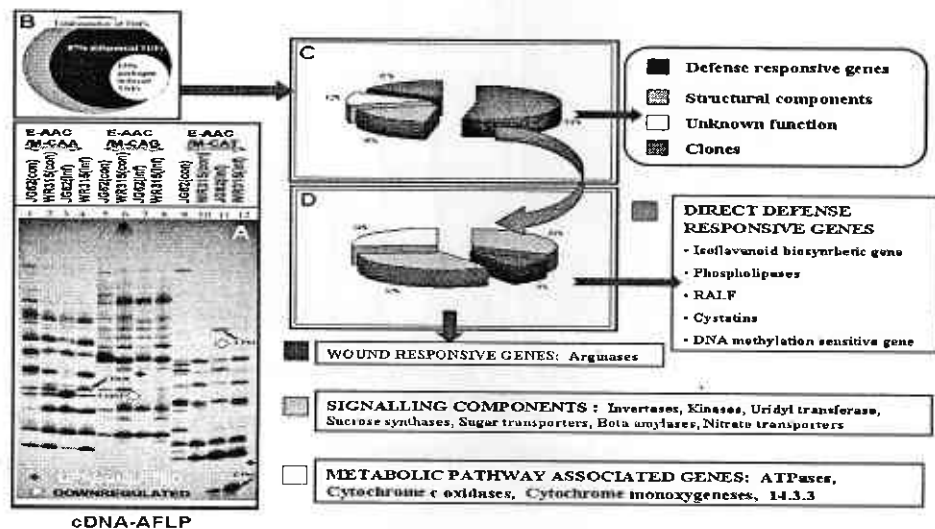

Fig. 5: A. cDNA-AFLP analyses showing upregulated and downregulated transcript derived fragments (TDFs). B. Percentage of TDFs differentially expressed during pathogen attack. C. Clustering of differentially expressed transcripts. D. Clustering of defense responsive transcripts related to direct defense, wounding, signaling and metabolic components (Adapted from Gupta *et al.* 2009).
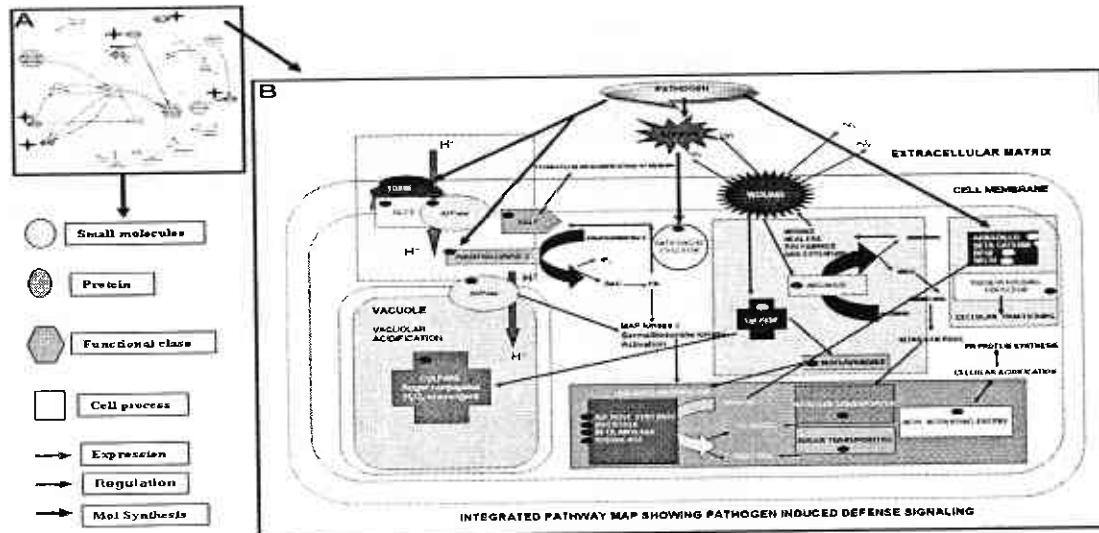
Fig. 6 : A. Network showing the interaction of five (marked in asterisks) defense responsive genes (homologues obtained from Arabidopsis thaliana database, TAIR) using Pathway Studio (version 7). B. Schematic representation showing the inter relationship between the defensive molecules and their downstream signaling. Marked components represent the identified transcripts from the chickpea-*Fusarium* case study (Adapted from Gupta *et al.* 2009, Gupta *et al.* 2010).

evolving MAMPs or PAMPs (Microbe/ Pathogen associated molecular patterns), such as bacterial flagellin or fungal chitin (Dodds and Rathjen 2010, Zipfel and Felix 2005) or DAMPs (Danger associated molecular patterns) such as cell wall or cuticular fragments or other endogenous molecules released by pathogen invasion (Dodds and Rathjen 2010). Stimulation of PRRs results in PAMP-triggered immunity (PTI) that can halt further colonization. Successful pathogens however deploy effectors that suppress PTI resulting in effector-triggered susceptibility (ETS) (Jones and Dangl 2006). Direct or indirect recognition of a given effectors by specific intracellular receptor leads to effector-triggered immunity (ETI). Since effectors are variable and dispensable natural selection drives pathogens to diversify the existent effector genes to avoid ETI or/and to evolve new ones to suppress ETI. Natural selection again comes into play and results in acquisition of receptors with new specificities by the host plant thus triggering further amplified ETI (Dodds and Rathjen 2010, Jones and Dangl 2006) (Fig 1). PTI and ETI generally give rise to similar responses, although ETI is qualitatively stronger and faster often giving rise to hypersensitive response.

Looking for key players in Plant Immune System

A proper understanding about the machinery adapted by plants to survive against the pathogen is essential for identifying key players in plant defense response. Advances in omic technologies have lead to generation of enormous amount of data about immune signaling and responses. It is now known that plant receptors on interaction with their cognate elicitors trigger complex signaling pathways leading to activation of defense associated proteins. However for a plant to be able to mount resistance it must recognize the invading pathogen at early stages of infection and must be able to rapidly activate defense pathways. The following case studies highlight the complexities of early defense in model cereal (rice) and legume (chickpea) crops

The Rice-Xanthomonas case study

Rice is a popular cereal crop grown in tropical, subtropical and temperate regions of the world. It is a major food crop of more than sixty percent of the world and contributes to fifty-two percent of total food grain production in India. Rice as a food source is very important in developing world, where it is often equated with food security. The changes in rice availability and its cost have far reaching social and political ramifications. Rice is affected by various biotic and abiotic factors resulting in huge gap between yield potential and actual yield. Bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) is a very devastating disease of rice.

It is distributed in all the major rice growing areas of the world including Asia, Africa, America and Oceania. It is a rod-shaped, round-ended, Gram-negative bacterium with a single polar flagellum. The bacteria enter through hydathodes or wounds on roots or leaves. Once inside the vascular system it multiplies inside the xylem vessels, completely clogging them. Bacterial leaf blight appears on leaves of rice plants as yellowish green or grey green water-soaked streaks near leaf tip or margins (Fig 2). These lesions expand and coalesce, eventually the whole leaf and then the whole plant is affected and dies. Availability of genomes of both rice and Xoo has opened up new avenues, including microarray technology, for studying host-pathogen interaction in both contenders.

In our present investigation Microarray technology was used to delineate the transcriptomic changes taking place in susceptible IR24, an elite indica cultivar and resistant (IET8585) genotypes of rice after inoculation with Xoo. IET8585 is an indica rice line often cultivated as BLB (bacterial leaf blight) resistant check and can withstand wide range of Xoo pathovars. Since early recognition is required for resistance, the study carried out by Grewal et.al (2012) focused on investigating the differential gene expression one hour after inoculation; 274 genes were found to be differentially expressed in resistant plant compared to susceptible ones, of these 112 were up-regulated and 73 were down-regulated in IET8585 as compared to IR24 (Grewal et al. 2012). The microarray results were validated using quantitative PCR analysis (Grewal et al. 2012). Through comparison of transcriptomic data and gene interaction maps it was found that the major up-regulated cluster constituted of cell signaling proteins and transcription factors while growth and basal metabolic components were largely down-regulated (Fig 3). Further the data suggested that as the plant faces the pathogenic challenge it suspends its growth till it can spare the resources. This study highlighted the complexity of defense pathways; calcium signaling, lipid signaling as well as MAPK cascade were found to be modulated by signals from surface and cytosolic R-proteins to arouse jasmonic acid and ethylene signaling and to suppress auxin signaling through transcription factors (Grewal et al. 2012). Components of primary as well as secondary metabolism were adjusted to mount appropriate defense responses. The gene modulations undertaken by plant cells at one hour after inoculation highlight the ability of plant cells to rapidly mount a complex defense response.

The Chickpea-Fusarium case study

Chickpea (*Cicer arietinum* L) is the most important pulse crop of Indian sub continent and ranks third in the list of internationally important pulse legumes (www.fao.org). The crop grows well in tropical, subtropical and temperate regions of the world (Muehlbauer and Singh 1987). It is valued greatly for its high nutritional content among which 25%-29% comprises of easily digestible protein. It is also considered important from the economical point of view especially in undernourished countries where high cost animal protein fails to reach the native mass. But every year about 10%-90% crop yield is lost due to fungal attack by wilt causing fungus *Fusarium oxysporum* f. sp *ciceri* (Foc) (Haware and Nene 1982). This soil or seed borne pathogen has two different pathotypes. The yellowing pathotype induces foliar yellowing with vascular discoloration while the more devastating wilt causing pathotype induces rapid chlorosis, flaccidity, and vascular discoloration (Haware and Nene 1982). The fungus colonizes in the xylem vessels and completely blocks the ascent of sap, thus resulting in wilting of the entire crop (Cho and Muehlbauer 2004). Eight pathogenic races of F. *oxysporum* f. sp. *ciceri* have been identified (races 0, 1, 1B/C, 2, 3, 4, 5, and 6) till date, of which races 1, 2, 3, and 4 are known to exist in India (Haware and Nene 1982). Races 0 and 1B/C induce yellowing of infected plants while the rest cause wilting. Unlike others, only race 1, known to have wide geographic distribution has received prime investigative concern in India as well as worldwide. Although, Fusarium wilt is primarily managed by implementing natural resistant breeding strategies, but unavailability of high yielding resistant cultivars, breakdown of natural resistance over period of time and generation as well as variability of pathovarieties strongly limit the progress of resistant breeding programs (Jiménez-Gasco et al. 2004). Hence, a comprehensive biochemical as well as molecular study of the interactions of resistant and susceptible chickpea cultivars with the pathogen is needed for developing effective breeding programs and producing cultivars with sustainable resistance. In addition, optimization of gene

transfer technology is also an essential prerequisite for developing effective broad-spectrum resistant crops which again require thorough understanding of the plant-pathogen inter-chemistry.

In an attempt to identify the molecular components inducing plant defense upon infection with F. oxysporum f. sp. ciceri race 1 (Foc1), experiments were designed to trigger disease responses in both susceptible and resistant plants and monitor the expression of stress induced genes/ gene fragment(s) at transcript level. Microscopy revealed in planta pathogenic establishment and their nature of progression within the host (Fig 4). cDNA-Amplified fragment length polymorphism (cDNA-AFLP) followed by homology search helped in differentiating and analyzing the up and down regulated gene fragments (Fig 5). Some of the important transcript derived fragments (TDFs) were homologous to genes related to early defense, wounding, secondary metabolism as well as several others linked to primary metabolism of the host (Gupta et al. 2009) (Fig 5). Reverse transcriptase polymerase chain reaction (rt-PCR) and real time PCR (qRT-PCR) analyses also confirmed the early recognition of wound inducing pathogen by the host. Networks of interacting pathways and cellular processes also showed that the interplay between fungus and host induced changes in primary metabolism and generated defense signals (Fig 6) in combating pathogenic encounter (Gupta et al. 2010) Besides, this study also highlighted the limitations of hypersensitive response mediated resistance especially when the central solute conducting machinery of the host plant is the primary site for biotrophic fungal colonization.

On the whole, with the results obtained so far the study predicts that in case of compatible interaction Foc1 establishes within the host, triggers HR, targets the host's primary metabolism and overpowers host resistance. Conversely, in case of incompatible interaction the pathogen is sensed early by the resistant plants, its establishment within the host is delayed, HR intensity is comparably lower than the susceptible variety and host primary metabolic signals somehow compensate for the pathogen-induced damage (Gupta et al. 2010). Thus, further characterization of all the identified defense responsive genes and their roles is needed to provide a better conclusive depiction of the plant-pathogen interaction study.

Improving Plant Innate Immunity

The plethora of information so far obtained suggests that plant defense response is a very complex phenomenon, involving differential regulation of many pathways and crosstalk amongst them at different levels. However it also provides the means to identify key regulators of plant defense and tools to manipulate these regulators to develop plant resistance. Receptors of conserved microbial signatures have been used for engineering broad-spectrum resistance into plants against pathogens that were not previously recognized (Fradin et al. 2009, Lacombe et al. 2010, Song et al. 1995); transfer of XA21 from Oryza longistaminata to Oryza sativa induced resistance to Ax21 containing Xanthomonads; overexpression of OsBAK1 in transgenic rice enhanced resistance to Magnoporthea oryzae (Li et al. 2009). In past attempts were also made to enhance plant immunity by overexpressing individual components of defense pathways (Stuiver and Custers 2001). Grapevine phytoalexin resveratrol biosynthetic pathway was engineered into tobacco and tomato (Hain et al. 1993, Thomzik et al. 1997). Overexpression of hydrolytic enzymes i.e. glucanases, chitinases have been used to enhance plant immunity in wheat and rice (Gómez-Ariza et al. 2007, Narasimhan et al. 2009, Shin et al. 2008). Antimicrobial peptides of biological or synthetic origin have also been introduced into transgenic plants (Marcos et al. 2008, Rahnamaeian et al. 2009). However these approaches have met with limited success since boosting of individual components of elaborate defense pathways result in relatively weak or highly narrow spectrum immunity (Gurr and Rushton 2005, Marcos et al. 2008, Stuiver and Custers 2001). But now, with accumulation of knowledge, designing strategies to boost the immune response in its entirety has become possible. NPR1, a key regulator of systemic acquired resistance (SAR), over-expression have been used in many crops to induce immunity (Chern et al. 2005, Makandar et al. 2006, Spoel and Dong 2008). Similarly constitutive expression of MAPKs or immunity associated transcription factors enhances broad-spectrum immunity (Century et al. 2008, Yamamizo et al. 2006).

CONCLUSION

Since success of a disease management strategy

depends upon the balance between pathogen control and effect on plant growth, in depth study of effects of an immunity booster on plant physiology is essential, which is now possible through proteomics and metabolomics. The genomic era has provided us with an enormous arsenal to design strategies against pathogens. It has provided us tools to look for novel receptors, which will be in continuous need as new pathotypes evolve. A better understanding of plant defense response have helped us to identify major regulators of plant defense response, which may be used to enhance systemic acquired resistance or hypersensitive response against necrotrophs or biotrophs as the case may be.

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