Importance and Use of Resistance Gene Analogs

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Abstract: The identification, isolation and subsequent cloning of effective disease resistance genes for their use to enhance the protection level against various groups of pathogens in plants is an important aspect of crop improvement. Disease resistance genes (*R*-genes) have been characterized and cloned from many plant species. The resistance gene analogs (RGAs) are putative or tentative disease resistance genes which are identified on the basis of their structure. The RGAs are an efficient tool in identifying and isolating disease resistance genes and have got efficiency in building a durable resistance. In order to know the exact function of RGAs they need to be characterized and linked with the genes actually conferring resistance phenotype. Regions of amino acid conservation in resistance gene encoded proteins have facilitated the isolation of RGA sequences from genomic DNA in many crop plants using polymerase chain reaction based techniques.

INTRODUCTION

Nature has blessed the crop plants with an inbuilt mechanism to defend themselves from the invasion of pathogenic organisms. This inherent inbuilt mechanism restricts the invasion and proliferation of potent pathogens and is termed as resistance. The genes responsible to confer resistance phenotype to the crop plants are called resistance genes. The term resistance gene analog originated in last decade with subsequent cloning and knowing the structure of disease resistance genes. Up till 1990, the protein products encoded by such genes were not known, but now a growing number of disease resistance genes conferring resistance to a diverse spectrum of pathogens have been isolated from a wide range of plant species and over 40 such genes have been cloned [1]. The first disease resistance gene cloned was *Hm1* in maize conferring resistance against leaf spot causing fungus *Cochliobolus carbonum*. This gene encodes a reductase which detoxifies fungal HC-toxin and rendering the plant resistant to the said fungus. After comparing the protein products encoded by different disease resistance genes, it was found that certain amino acid sequences remain conserved. The idea of using these conserved sequences of different disease resistance genes to design primers for PCR amplification to find such similar sequences in genomic DNA, initiated the hunt for such perfect markers. So resistance gene analogs are the amplification products got through the PCR amplification of genomic DNA using primers designed from the conserved sequences of known disease resistance genes.

It was Biffen [2] who for the first time established that like all other traits disease resistance is an inherited trait. His study was based on the stripe rust resistance in wheat cultivar RIVET for simple Mendelian Inheritance. He simply crossed resistant and susceptible plants and found F_2 population segregating in the ratio of 1 Resistant: 3 Susceptible. Such observations lead him to conclude that resistance against stripe rust fungus in wheat cultivar RIVET is controlled by single recessive gene. Biffen's study opened way and established the future of genetics of disease resistance.

The genetics of host-pathogen interaction system was studied by H. H. Flor [3]. Based on the studies on rust resistance in linseed (*Linum usitatisimum* L.) he proposed the gene-for-gene hypothesis [3]. According to this hypothesis, for every gene conditioning resistance in the host there is a corresponding gene for avirulence in the pathogen. This hypothesis changed the concepts of plant breeders working on host-pathogen interaction systems and insisted them to study both genetics of host as well as the pathogen. In other words, this hypothesis revolutionized the genetics and plant breeding aspects of disease resistance by compelling the plant breeders to do evolutionary and migration study of pathogen while investigating the genetic basis of disease resistance in host. Gene-for-gene hypothesis since last about 50 years of its discovery still holds true for understanding the genetics of host-parasite systems. Investigations for the study of host-parasite systems at the cellular level have revealed that the resistance gene encoded proteins (receptors) act as sensors on and inside the cell membrane [4]. Whenever these resistance gene products come in contact with their specific pathogen gene product (ligand/elicitor), the signal is transferred inside the cell. This signal transfer leads to ionic imbalance, increase in ca^{2+} ions in cytoplasm, production of reactive oxygen species (ROS like; H_2O_2, Nitric oxide) and salicylic acid. Production of ROS in the cytoplasm has not only been found in plants but also in animals like human beings under the stress conditions. In order to reduce these ROS and bring the plants to normal condition, biochemists suggest the application of peroxidases and catalases. Any of

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the above conditions present in the cell stimulates the defense response genes. This defense response is often in the form of localized cell death (LCD) caused by the production of phytoallexins [5]. This receptor-ligand interaction model holds true for fungal, bacterial, viral as well as in insect related resistance gene responses.

CLONING OF DISEASE RESISTANCE GENES

Once the identification and mapping of the effective disease resistance genes is achieved, the next step is their cloning. Cloning simply means increasing the copy number of genes. There is a need to have many copies of resistance gene fragment encoding for a complete open reading frame, due to two main reasons.

- After identifying disease resistance genes from the source varieties, there is need to transfer these genes to other varieties of the crop (homologous system), across species or genera (heterologous system), where protection is needed from similar pathogens. So the geneticists need to have many copies of genes to exploit or in other words intentionally spread those copies of disease resistance genes across species and genera infected by similar group of pathogens.
- Certain disease resistance genes have been found which are capable of recognizing more than one type of *avr* proteins (avirulent proteins expressed by the pathogen). Such genes have been well identified from crops like; Wheat (*Lr*34,*Sr*2)- [6,7], Maize (*RP*1)- [6,8], Tomato (*Pto*)- [9], Barley (*Mla*)- [10], Pepper (*Bs*2)- [6,11], Arabidopsis (*Npr*)- [6,1] etc**.** It was also found that increased copy number alters the level of expression of certain resistance genes where resistance is associated with constitutive defense response.

Such methods of engineering broad spectrum resistance are durable, since it is independent of an interaction with a specific *avr* gene [6]. Through many studies it has also been found that a combination of few race specific and race nonspecific disease resistance genes in the host are effective in building a type of resistance, which is durable [12].

CLONING METHODS

Many methods have been used for cloning of disease resistance genes. There are some general as well as some crop specific techniques used for isolation and copying the disease resistance genes. Two methods which have been mostly used are:

- Transposon tagging
- Map-based cloning

Transposons are the small DNA (Deoxyribonucleic acid) sequences capable of changing their place in the genome by virtue of the enzyme transposase encoded by them. Taking advantage of their ability of de-functioning a gene, various transposons have been linked to different genes they are capable to recognize. This process is referred as transposon tagging.

For map-based cloning of resistance genes we need to develop a saturated genetic map. More the number of markers applied in mapping, more is the probability of getting a closely linked marker. We need yeast artificial chromosome (YAC) library to get first a larger DNA fragment of several hundred kilo bases spanning the resistance gene encoding region. Then we need to screen a cosmid library bearing genomic DNA fragments of smaller size, to identify the cosmid clone having gene of interest. The ultimate aim of map-based cloning is to identify a smallest DNA fragment encoding a complete open reading frame (ORF). The identified clone is then transformed into the susceptible cultivar for confirmation of a resistant phenotype. Many disease resistance genes have been cloned using these techniques. At transposon tagging we look for a susceptible plant where the transposon has been inserted into resistance gene to defunction it. Using transposon specific sequences we need to amplify the flanking sequences using inverse PCR (polymerase chain reaction). These flanking sequences can then be used for isolation and copying of disease resistance gene. Even though many resistance genes have been cloned using transposon tagging, map-based cloning has an edge over it and is considered as prime choice. Presence of large fraction of repetitive DNA (up to 80%) in cereal genomes has been recognized as one of the major hurdle in map-based cloning of genes. Under such situations certain crop specific approaches like diploid/polyploid shuttle mapping strategy have been successfully used; like that of cloning of leaf rust resistance gene *Lr*21 [13].

STRUCTURE OF DISEASE RESISTANCE GENES

After cloning analyzing the structure of disease resistance genes has revealed that the genes conferring resistance against fungal, bacterial and viral group of pathogens have got certain structural similarities. The most notable conserved amino acid motifs within different classes of resistance genes are the nucleotide binding site (NBS) and leucine rich repeat (LRR) regions. NBS plays important role in signal transduction pathway, while LRR regions are concerned with pathogen recognition or protein-protein interaction. NBS-LRR genes are abundant in plant genomes, with approximately 150 described in Colombia ecotype of *Arabidopsis* [14] and about 500 estimated in rice genome [15].

CLASSIFICATION OF DISEASE RESISTANCE GENES

Large numbers of disease resistance genes are known to confer resistance against invasion of diverse pathogenic organisms. Hammond-Kosack and Parker [16] grouped these resistance genes into different classes on the basis of the predicted features of resistance gene encoded proteins (Table **1**).

SYNTHESIS OF DEGENERATE PRIMERS

By sequence comparisons within different classes of cloned disease resistance genes certain conserved amino acid motifs have been identified. These conserved sequences have been used for synthesis of degenerate primers to be used for PCR amplification of genomic DNAs from diverse crop species. Degenerate primers are the primers synthesized from conserved sequences which differ for few nitrogenous bases from each other.

ISOLATION OF RGAS

The strategy of using primers synthesized from conserved motifs of resistance genes to amplify resistance gene

Table 1. The Major Classes of Cloned Plant Disease Resistance Genes

Class	Gene	Plant	Pathogen	Infection Type/ Organ Attacked	Predicted Features of R protein*	Race Specific	Year Isolated	References
1	Hm1	Maize	*Helminthosporium maydis* (race 1)	Fungal necrotroph/leaf	Detoxifying enzyme HC toxin reductase	Yes	1992	[17]
2	Asc-1	Tomato	*Alternaria alternata* f.sp. *lycopersici* (AAL toxin)	Fungal necrotroph/leaf	TM helix-LAG1 motif	No	2000	[18]
3A	Pto	Tomato	*Pseudomonas syringae* p.v. tomato (*avrPto*)	Extracellular bacteria/leaf Intracellular serine/ threonine protein	Kinase	Yes	1993	[17]
3B	PSB1	Arabidopsis	*Pseudomonas syringae* p.v. *phaseolicola* (*avrPphB*)	Extracellular bacteria/leaf	Different subfamily	Yes	2001	[19]
4A	RPS2	Arabidopsis	*Pseudomonas syringae* p.v. *maculicola* (*avrRpt2*)	Extracellular bacteria/leaf	CC-NB-LRR Intracellular protein	Yes	1994	[17]
	Mla1/ Mla6	Barley	*Blumeria graminis* f.sp. *hordei* (resp. race 1, race 6)	Biotrophic intracellular fungus with haustoria/leaf		Yes	2001	[20]
	R1	Potato	*Phytophthora infestans* (race 1)	Biotrophic intracellular Oomycete with haustoria/leaf and tuber		Yes	2002	[21]
	RPP8	Arabidopsis	*Peronospora parasitica*	Biotrophic intracellular Oomycete with haustoria/leaf		Yes	1998	[17]
4B	N	Tobacco	Mosaic virus	Intracellular virus/leaf and phloem	TIR-NB-LRR Intracellular protein	Yes	1994	[17]
	RPP4	Arabidopsis	*Peronospora parasitica*	Biotrophic intracellular Oomycete with haustoria/leaf		Yes	2002	[22]
4C	Bs2	Pepper	*Xanthomonas campestris* pv. *vesicatoria* (*avrBs2*)	Extracellular bacteria/leaf	NB-LRR Intracellular protein	Yes	1999	[17]
	Dm3	Lettuce Bremia	lactuca	Biotrophic intracellular Oomycete with haustoria/leaf		Yes	2002	[23]
4D	RRS-1	Arabidopsis	*Ralstonia solanacearum* (race 1)	Extracellular bacteria/leaf	TIR-NB-LRRNLS-WRKY	Yes	2002	[24]
4E	Pi-ta	Rice	*Magnaporthe grisea* (*avrPita*)	Hemibiotrophic intracellular fungus without haustoria/leaf	NB-LRD	Yes	2000	[17]
5A	Cf-9	Tomato	*Cladosporium fulvum*(*Avr9*)	Biotrophic extracellular fungus without haustoria/leaf	eLRR-TM-sCT Extracellular protein with single membrane-spanning region and short cytoplasmic C terminus	Yes	1994	[17]
5B	Ve1 Ve2	Tomato	*Verticillium albo-atrum*	Extracellular vascular wilt fungus without haustoria/root and stem	CC-eLRR-TM-ECS eLRR-TM-PEST-ECS	Yes	2001	[22]
6	Xa-21	Rice	*Xanthomonas oryzae* p.v. *oryzae* (all races)	Extracellular bacteria/leaf	eLRR-TM-kinase	Yes	1995	[17]
	FLS2	Arabidopsis	Multiple bacteria (flagellin)	Extracellular bacteria/leaf		No	2000	[17]
7	RPW8.1 RPW8.2	Arabidopsis	Multiple powdery mildew species	Biotrophic intracellular fungus with haustoria/leaf	Small, probable membrane protein with CC domain	No	2001	[25]
8	Rpg1	Barley	*Puccinia graminis* f.sp. *tritici*	Biotrophic intracellular fungus with haustoria/stem	Receptor kinase-like protein with 2 tandem kinase domains	No	2002	[26]

*ECS= endocytosis signal; LAG1= longevity assurance gene; LR= leucine-rich domain; PEST= Pro-Glu-Ser-Thr; sCT= single cytoplasmic tail; TM= transmembrane Hammond-Kosack and Parker [16].

analogs has been successfully used to isolate and map RGAs in various crop species like; Rice [15, 27, 28], Wheat [29, 30], Soybean [31], Barley [10], Cotton [32], *Arabidopsis* [1, 33, 34], Pepper [35], Potato [36]. Recently, Cloning and characterization of resistance gene analogs from under exploited plant species was reported [37], while Kozjak *et al.* [38] could develop 11 RGA markers, out of which eight amplified PCR products of expected sizes and two RGA markers segregated in the mapping family. Once the RGAs are isolated and mapped on to a particular chromosome, the next step is the cloning of RGAs.

CLONING OF RGAS

From various crop species genomic DNA could be isolated for its use in PCR amplification by using primers designed from conserved sequences of cloned disease resistance genes like; *Xa*21, *Pto*, *L6*, *N*. These primers called degenerate primers usually differ for few base pairs so as to amplify different RGA loci across the whole genome. The amplified products are separated on agarose gel for examination and conclusion of the results. The PCR products are then cloned in vector and such clones are called RGA clones. These RGA clones are then sequenced and the deduced amino acid sequences of the RGA clones are then aligned with that of known disease resistance genes to observe their percent homology with them.

CHARACTERIZATION OF RGAS

After knowing the proteins encoded by disease resistance genes, it was possible to synthesize primers (on the basis of their conserved sequences) to amplify RGA loci across whole genome. For mapping the RGAs the genomic DNA is digested with different restriction enzymes followed by electrophoresis and southern blotting of the product. Southern hybridization is done using RGA clones for detection of restriction fragment length polymorphism (RFLP) markers. RGAs mapping within or close to resistance genes and encoding homologous amino acid sequences can be used as candidates for those resistance genes.

IMPLICATIONS OF RESISTANCE GENE ANALOGS

RGAs represent potentially useful genes, containing conserved sequences like LRR, NBS, Kinases. Rice genome is smallest among the cereal genomes (450Mb) and consists of 12 chromosomes. The sequences of interest in the rice genome are the resistance genes which activate hypersensitive response (HR) in plants. The three key properties of resistance gene products are: Pathogen recognition; The recognition of corresponding *avr* gene related ligand; Signaling cascade activation through kinases and capacity for rapid evolution of specificity. These qualities result in several common structural classes of resistance genes. The availability of rice genome sequence enabled the global characterization of the largest class of plant disease resistance genes i.e., the genes encoding NBS-LRR sequences. From various studies it was found that the rice genome carries approximately 500 NBS-LRR genes that are very similar to the non-Toll/interleukin-1 receptor homology region (Class 2 genes of *Arabidopsis*) but none of that are homologous to TIR class genes. Over 100 of these genes were predicted to be pseudogenes in rice cultivar Nipponbare, but some of these are functional in other rice lines. Over 80 other NBS encoding genes were identified that belong to four different classes, only two of which are present in dicotyledonous plant sequences present in databases. Map positions of the identified genes show that these genes occur in clusters, many of which included members from distantly related groups. Members of phylogenetic subgroups of class 2 NBS-LRR genes mapped to as many as ten chromosomes. The patters of duplication of the NBS-LRR genes indicated that they were duplicated by many independent genetic events that have occurred continuously through the expansion of the NBS-LRR superfamily and the evolution of the modern rice genome. Genetic events such as inversions that inhabit the ability of the recently duplicated genes to recombine and promote the divergence of their sequences by inhabiting concerted evolution [15]. Koczyk and Chelkowski [28] using BLASTP and Hidden Markov Model searches to find the similar sequences as that encoded by different resistance gene analogs found that most of CC-NBS-LRR, CC-NBS, NBS-LRR and NBS map on to chromosome 11 of rice. They also found that the sequences homologous to *Pto*, *Xa21*, and *Cf9* type resistance genes are present on chromosome 1. Monosi *et al.* [15] from similar type of studies could find that there are approximately 500 NBS-LRR type genes in rice genome and these domains form clusters. From the above three studies it was concluded that; out of 106 known disease resistance genes in rice 28 are present on chromosome 11; RGA sequences may encode proteins having function other than disease resistance (stress related genes); TIR-NBS-LRR genes evolved after the divergence of monocotyledonous and dicotyledonous crop species and clustering of RGAs occurs due to their origin from common evolutionary mechanism. That is, common evolutionary forces are responsible for evolution of such RGA sequences. The results of Chelkowski and Koczyk [1] on completely sequenced genome of *Arabidopsis* (120Mb) using database searches like; BLASTP and Hidden Markov Model based searches were as; putative resistance genes identified from *Arabidopsis thaliana* can be of use in searching similar sequences in monocots; *Arabidopsis* genome contains both TIR-NBS-LRR and CC-NBS-LRR type genes; in cereals genome TIR motifs are altogether absent and CC-NBS-LRR type genes are common to both monocotyledonous and dicotyledonous crop species; studies on defense response genes must be under taken and sequence compared at protein level and amplification products are surely involved in signal transduction pathways in plants**.** As the resistance gene analogs encode the amino acid sequences which take part in transfer of signal to activate the defense response. Different domains perform different functions to have an ultimate response in the form of resistance phenotype. The nucleotide binding site (NBS) involved in signal transduction; leucine rich repeats (LRR) involved in protein-protein interaction; toll and Interleukin receptor homologue (TIR) involved in signal transduction; coiled coil (CC)/ leucine zipper (LZ) take part in formation of protein dimmers involved in signal transduction and kinases take part in phosphorylation, that is, if protein kinases are present they take

part in protein phosphorylation [39]. Hanan *et al.* [40] studied the diversity patterns of 204 markers derived from two *R*-gene domains, nucleotide binding site (NBS) and leucine-rich repeat (LRR) and demonstrated that the RGA profiling is an excellent tool for studying diversity of *R* genes in natural plant populations.

RESISTANCE GENE ANALOGS AND MAPPING OF RESISTANCE GENES

Mapping of RGAs generates perfect markers which are linked to known resistance genes. Various studies in which different resistance gene analogs have been used to follow resistance genes in segregating populations, link them and then ultimately use them as candidates for gene isolation. Collins *et al.* [10] tried to link some resistance gene analogs with different rust resistance loci in Barley. They were successful in mapping most of the resistance gene analogs within or close to the rust resistance gene loci. Genes can be isolated from species from which no disease resistance genes have yet been cloned. Based on the comparative mapping of Rice, Barley and Foxtail millet scientists could find some synteny across the three genomes [41]. Such study used some RGAs as probes which confirmed the existence of some conserved sequences across genomes. They concluded that these resistance gene analogs can be used to study comparative genomics in cereals as well as to compare cereals with other crop species. In some crop plants non-availability of saturated genetic maps makes map-based cloning of disease resistance genes difficult. Using RGAs even in such cases, resistance genes can be perfectly tagged and then cloned for their exploitation in heterologous systems. The crops which do not normally flower, or were the breeders face difficulty in crossing and the crops with high chromosome number like that of Sugarcane non availability of saturated genetic maps makes the problem much complicated as genetic studies are very difficult to conduct. RGAs provide an alternative of identifying and isolating disease resistance genes even in such crops. RGA sequences may sometimes contain stop codons resulting from nucleotide deletions, insertions or substitutions. Such genes correspond to certain inactive genes called pseudogenes. Kanazin *et al.* [31] isolated certain resistance gene analogs in soybean and compared them with known disease resistance genes *N* and *L6*. The RGAs isolated need to be compared with already known disease resistance genes at amino acid level. As different triplet codons may encode for same amino acid, so amino acid level comparison has an advantage over DNA sequence based comparison. The gaps in the amino acid sequences belong to the pseudogenes, which are capable of accumulating large number of mutation and ultimately are the sources for new resistance genes.

RESISTANCE GENE ANALOGS AND EVOLUTIONARY STUDIES

Scherrer *et al.* [29] used resistance gene analogs to study the evolution of two haplotypes existing on chomosome 1A of hexaploid wheat. The haplotype1 (H1) having complete RGA sequences (rga1& rga2), while haplotype 2 (H2) had only small portion of RGA sequences. The conclusion was based on the genotypes of *Triticum monococum* (A-genome donor) and wild and cultivated tetraploid wheats *Triticum dicoccoides* and *Triticum durum* (both AB-genome donor). They could find only H1 haplotype and no H2 haplotype in tetraploid wheats. They concluded that the H2 haplotype in hexaploid wheats is an direct introgression from A-genome donor *Triticum monococcum*.

CONCLUSION AND FUTURE DIRECTIONS

Resistance gene analogs are putative or tentative disease resistance genes identified on the basis of their structure. In order to know their exact function they need to be characterized and linked with the genes actually conferring resistance phenotype. A well known CIMMYT scientist Dr. R P Singh at the 4th International crop science congress 2004 [12] suggested three important strategies for safe guarding crops in future i.e., Study of evolution and migration of pathogen, Study of genetic basis of disease resistance and Identification and deployment of genes conferring durable resistance. The RGAs are an efficient tool in identifying and isolating the disease resistance genes and have got efficiency in building durable resistance. Earlier plant breeding approaches were targeted towards enhancement of yield, and then the objectives were shifted towards disease resistance and quality improvement. Today's plant breeding is an integrated approach involving all those three important objectives. Under such situation more efficient tools are needed to increase the efficiency of research, use of RGAs as candidates for disease resistance genes is one of such approach.

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ABBREVIATIONS

avr	=	Avirulence protein
CC	=	Coiled coil
CIMMYT	=	International maize and wheat improvement centre
DNA	=	Deoxyribonucleic acid
eLRR	=	Extracellular leucine-rich repeat
LRR	=	Leucine-rich repeat
LCD	=	Localized cell death
NBS	=	Nucleotide binding site
ORF	=	Open reading frame
PCR	=	Polymerase chain reaction

R = Resistance protein

RFLP = Restriction fragment length polymorphism

RGA = Resistance gene analog

ROS = Reactive oxygen species

TIR = Toll and Interleukin-1 receptor

YAC = Yeast artificial chromosome

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