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Two and a half decades of plant R-gene studies: A review

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Abstract
Resistance genes (R-Genes) are genes in plant genomes that convey disease resistance to plants against pathogens by producing R proteins. Food and Agriculture Organization, UN, defined R genes as "a class of plant genes conferring resistance to a specific strain of a particular pathogen primarily by sensing the presence of the pathogen and triggering the defense pathways in the plant". Plant R-genes involved in gene-for-gene interactions with pathogens are expected to undergo co-evolutionary arms races in which plant specificity and pathogen virulence continually adapt in response to each other. Plants evolved disease resistance (R) proteins to specifically detect the presence of the pathogen effectors called avirulence factors (Avr) once recognized by R proteins and subsequently trigger a much stronger defense response to counter the suppression of the Microbe Associated Molecular Pattern (MAMP) triggered immunity (MTI). It has been more than 25 years of R gene isolation, cloning and characterization and rigorous reviews have revealed the functional mechanisms underlying resistance at molecular level but whatever is known till date is merely a tip of the iceberg.

Keywords: R gene, NBS-LRR, evolution, R-Avr interaction, R gene classes, executor

Introduction
Plants like all other living organisms on earth need to defend themselves against attack from various pathogens like fungi, viruses, bacteria, invertebrates and even other parasitic plants. Since plants lack a circulatory system, each plant cell must possess a preformed and/or inducible defense capability, which also distinguishes plant defense system from the animal immune system (Walbot, 1985) [1]. Plant diseases can drastically subside the crop yields as the degree of disease outbreak is getting severe around the world. Therefore, plant disease management has always been one of the main objectives of any crop improvement program (Gururani *et al*., 2012) [2]. Despite substantial advances in plant disease control strategies, the global food supply is still threatened by a multitude of pathogens and pests (Garelik, 2002) [3]. According to Agrios (2005) [4], R genes enable the plant to remain resistant to pathogens carrying the corresponding avirulence (avr) genes. Plant innate immunity mainly depends on cell surface receptors outside the cells and on nucleotide binding sides (NBS) and leucine rich repeats (LRR) inside cells. Most of the plant disease-resistance genes (R-genes) cloned hitherto code for NBS-LRR proteins (Jones and Dangl, 2006; Jones *et al*., 2016) [5, 6]. Numerous R genes have been identified, cloned and characterized in plants so far, e.g. roughly 165 R genes have been identified in *Arabidopsis thaliana* (Shao *et al*., 2016; Gao *et al*., 2018) [7, 8]. Recently three types of Transcription activator-like (TAL) effector associated R genes have also been characterized during *Xanthomonas*/plant interaction studies, which imparts recessive, dominant non-transcriptional and dominant TAL effector-dependent transcriptional based resistance as reviewed by Zhang *et al*., (2015) [9]. The constructs of plant R-gene products i.e. R- proteins are characterized by an N-terminal signaling domain, a nucleotide binding adaptor and CED-4 (NB-ARC) domain, and a series of LRRs (Urbach and Ausubel, 2017) [10]. Comparative genomic analyses suggests wide distribution of R-genes in land plants (Shao *et al*., 2014; Jones *et al*, 2016; Urbach and Ausubel, 2017) [11, 6, 10]. However, no R-gene has been reported in algae yet. Thus, plant R-genes were proposed to originate in land plants (Yue *et al*., 2012; Urbach and Ausubel, 2017) [12, 10]. Several R-gene analogs are fixed in plant species and are believed to contribute to non-host resistance in those plants (Schulze-Lefert and Panstruga, 2011) [13].

Phyto-Pathogen interactions and the genetic basis of plant defense
Plants unexceptionally share their own space with myriads of microbes (Spanu and Panstruga, 2017)[14]. In the case of living plants, this may result in apparently neutral (Shaw *et al*. 2016) [7], conjointly beneficial (Manck and Requena, 2016) [15] or detrimental (Langenbach *et al*. 2016) [16]

interactions and the respective microbes are commonly called as endophytes, symbionts and pathogens, respectively. Phyto pathogens are known to set out one of three main strategies to attack plants either necrotrophy, biotrophy, or hemibiotrophy. Necrotrophs are those which first kill host cells and then metabolize their contents. Some have wide host range, and cell death is often induced by toxins and/or enzymes targeted to specific substrates (Walton, 1996) [17]. Commonly known examples of fungal necrotrophs include *Pythium* and one the most dreadful *Botrytis* species (Shaw *et al*., 2016) [18]. For the necrotrophs, plant resistance can be achieved via the loss or alteration of the toxin's target or through detoxification of the pathogen toxins. Pathogen virulence is dominant because of the need to produce a functional toxin and/or enzyme, whereas avirulence i.e. the inability to cause disease, is inherited as a recessive trait. The first R gene isolated was Hm1 from maize, which confers resistance to the leaf spot fungus *Cochliobolus carbonum.* Hm1 encodes a reductase enzyme which detoxifies the *C. carbonum* HC-toxin that inhibits histone deacetylase activity (Johal and Briggs, 1992) [19] and the Hm1 gene product confers resistance through detoxification of this toxin.

Biotrophic and hemibiotrophic pathogens invade living cells and disrupt metabolism to favor their own growth and reproduction (Agrios**,** 2005) [4]. In simple terms, when the plants remain alive during the nutrient exchanges, the interaction is referred as biotrophic interactions and microbes as biotrophs (Spanu and Kamper, 2010) [20]. This is classically the case in symbiotic relationships, but also in some instances of parasitism. The frequent formation of "green-islands" on senescing leaves surrounding the biotrophic infection sites of fungal rusts and mildews attests to the importance of keeping host cells alive throughout this intimate association. Biotrophs are inclined to cause disease on only one or a few related plant species. In contrast, hemibiotrophic fungi such as *Phytophthora* and *Colletotrichum* kill surrounding host cells during the later stages of the infection. Therefore in such biotrophic/hemibiotrophic associations incompatibility frequently results in the activation of plant defense responses, including localized host cell death, the hypersensitive response (HR) (Hammond and Jones, 1996) [21]. In the 1940s, using flax (*Linum usitatissimum*) and its fungal rust pathogen *Melampsora lini*, HH Flor studied the inheritance not only of plant resistance, but also of pathogen virulence (Flor, 1971) [22]. His work revealed the classic "gene-for-gene" model that proposes that for resistance to occur, complementary pairs of dominant genes, one in the host and the other in the pathogen, are required. A loss or alteration to either the plant resistance (*R*) gene or the pathogen avirulence (*Avr*) gene leads to disease (compatibility), and this simple model holds true for most biotrophic pathogens, including fungi, viruses, bacteria, and nematodes. The discovery that plants have centers of origin, where the greatest genetic diversity resides and have co-evolved with pathogens, urged a series of breeding programs to identify resistant germplasm in wild relatives of crop species and followed by their introgression for agricultural benefit. Now, after more than two and a half decades of R gene cloning, mechanistic developments in the function of R gene products have been determined. Kourelis and van der Hoorn, (2018) [23] identified 314 cloned functional R genes through a comprehensive review and concluded that a functional mechanism has been proposed for 128 out of the 314 identified R gene products.

Properties of R Genes and Their Products

The dominant nature of *R* and *Avr* genes has led to the implication that *R* genes encode proteins that can recognize *Avr*-gene-dependent ligands. Following pathogen recognition, the R protein is presumed to activate signaling cascade(s) that coordinate the initial plant defense responses to impair pathogen ingress. Imbedded in this view is the notion that R proteins would be expressed in healthy, unchallenged plants in readiness for the detection of attack. A third requirement of R proteins is the capacity for rapid evolution of specificity. Frequently new virulent races of pathogens regularly evolve that evade specific *R* gene–mediated resistance (Crute and Pink, 1996; Michelmore, 1995) [24, 25]. Thus a mechanism is required by which plants can quickly evolve new *R* genes to resist virulent isolates. R genes encode putative receptors that respond to the products of 'Avr genes' (Avr, avirulence) expressed by the pathogen during infection. In several cases, a single R gene is capable of providing complete resistance to one or more strains of particular pathogen, when transferred to a previously susceptible plant of the same species. For this reason, R genes have been used in conventional resistance breeding programs for decades (Young, 2000) [26]

Classes of R Gene Proteins

R gene proteins have been categorized into 6 different classes on the basis of their function and location in the host cells. These R gene proteins consist of domains like Nucleotide Binding Site (NBS), Leucin Rich Repeat (LRR), Toll like Interleukin (TIR) and Coiled Coil (CC) domains, which are either extracellular, membrane associated, trans membrane or cytoplasmic. Most of the disease resistance genes (R genes) in plants cloned to date encode nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins characterized by nucleotide binding site (NBS) and leucine-rich repeat (LRR) domains as well as variable amino- and carboxy-terminal domains (McHale *et al*., 2006) [27]. These large, plentiful, proteins are involved in the detection of varied pathogens, including bacteria, viruses, fungi, nematodes, insects and oomycetes. LRR domains are the major determinants of recognition specificity for *Avr* factors. LRR regions are receptor domains for specific recognition of pathogen elicitors and may be involved in direct protein-protein interactions with *Avr* gene products of the pathogen (Bergelson *et al*., 2001)[28]. Besides these classes reports have also mentioned about existence of other classes of R gene protein due to differences in their structure like the enzymatic R-genes which contain neither LRR nor NBS groups, e.g. the maize *Hm1* gene against southern corn leaf blight and encodes the enzyme HC toxin reductase, which detoxifies a specific HC toxin (Gururani *et al*., 2012) [2].

Table 1: Classes of Plant R Gene Proteins

Class	Function	Example(s)
I	Membrane associated, mediating broad-spectrum resistance	RPW8
II	Cytoplasmic signal-transducing serine–threonine protein kinase	P*to*
III	Extracellular LRRs with transmembrane anchor	C*f-2–Cf-9*
IV	Extracellular LRRs, with a transmembrane receptor and a cytoplasmic serine–threonine kinase	Xa21
V	Cytoplasmic, membrane associated with LRRs, NBS and TIR domains	RPP5, N1, L66
VI	Cytoplasmic, membrane associated. Contain LRRs, NBS and a CC domain	RPM1, RPS2

Mechanism of Recognition of Pathogen Avirulence Gene by R Gene

Plant innate immunity consists of preformed physical and chemical barriers (such as leaf hairs, rigid cell walls, pre-existing antimicrobial compounds) and induced defenses. Successful invasion of preformed barriers by microbes may be recognized by the plant, resulting in the activation of cellular defense responses that stop or restrict further development of the invader. Two evolutionarily interrelated mechanisms have evolved in plants for detection and recognition of the invading microbes: First mechanism suggests that plants are able to recognize some conserved microbe derived molecules which are collectively described as microbe-associated molecular pattern (MAMP) by cell-surface receptors and trigger immune response which give rise to non-host resistance. Second mechanism plants evolved disease resistance (R) proteins to specifically detect the presence of the pathogen effectors called a virulence factors (Avr) once recognized by R proteins and subsequently trigger a much stronger defense response to counter the suppression of MAMP Triggered Immunity (MTI) by the pathogen (Xiao *et al.*, 2008) [29].

Evolution of R Gene

Plant R-genes involved in gene-for-gene interactions with pathogens are expected to undergo co-evolutionary arms races in which plant specificity and pathogen virulence continually adapt in response to each other (McDowell and Woffenden, 2003) [30]. The evolutionary mechanism of R gene is well explained by Agrios (2005) [4]. It is thought that when a plant was first attacked by a new pathogen strain, the plant probably had some genes encoding nonspecific receptor molecules that enabled the activation of defense responses to wounding and to pathogens in general but that it lacked any R genes to the new pathogen This pathogen, therefore, was able to cause considerable damage to the plant and possibly killed many of the susceptible plants. Plants exhibiting greater or lesser general resistance survived and multiplied to proportional extents. When, during the evolutionary race for survival of the plant from the pathogen, a resistance (R1) gene evolved, e.g., by modification of one of the general resistance genes, and that gene allowed the plant to recognize one of the initial steps of infection by the new pathogen (race 1) and to resist infection, such an individual plant and its progeny (variety 1) were selected for survival and so the plant and the R1 gene survived and multiplied. This might have happened, for example, by modification of one of the receptors involved in activating plant defenses against pathogens in general. Thus, the modified receptor 1 product of the R1 gene recognizes specifically a particular compound (elicitor 1) produced by a pathogen gene, which gene, as a result, behaves like an avirulence (*avr1*) gene. Pathogens carrying this *avr1* gene (race 1) cannot survive on such R1 gene-carrying plants. If, however, in time, a mutation affects the *avr1* gene of race 1 of the pathogen, which gene until now was the cause of its avirulence, the gene and the avirulence are destroyed. As a result, the new offspring of the pathogen become virulent again, capable of attacking the so-far resistant variety 1 of the plant. This new virulent pathogen population could be termed race 2. The host plant (variety 1) is now susceptible to race 2, which infects and may kill many plants. Soon, however, through survival pressure and selection, an R2 gene evolves that encodes a new or further modified receptor 2 that recognizes a different compound (elicitor 2) produced by the *avr* gene of individuals of the pathogen race 2. This gene, then, becomes the *avr2* gene conferring a virulence to the pathogen because it is recognized by the R2 gene of the plant. In this way, numerous, diverse R genes have evolved in a plant host to counteract corresponding virulence genes in the various races of one of its pathogens. The evolutionary process just described is supported by the fact that most of the R genes studied so far seem to be present in tandem arrays of multiple (up to 10 or more) related R genes: They exhibit different specificities but behave as though they are alleles of a single gene that cannot be separated during recombination or exist as a clustered gene family. This gene-for-gene interaction has occurred in a stepwise fashion over time and continues to date. It has also been reported that selection during domestication favored dominant R-genes providing full resistance, but recessive R-genes and R-genes that provide partial resistance may provide more durable resistance (Kourelis and van der Hoorn, 2018) [23].

R-AVR Interaction and Possible Functional Mechanisms Involved In Resistance

Generally but not always, in the host the genes for resistance are dominant (e.g. Xa1, Xa4, and Xa21), whereas genes for susceptibility, i.e., lack of resistance, are recessive (r). However, in the pathogen genes for avirulence, i.e., inability to infect, are generally dominant (A) whereas genes for virulence are recessive (a). Cao *et al* (2018) [31] have reported differential functional mechanism leading to cell death in the host plants while studying three dominant major resistance (MR) genes Xa1, Xa4, and Xa21 and two recessive MR genes xa5 and xa13 that encode quite different proteins and revealed that Xa1, Xa4, and Xa21 mediated resistances to *Xanthomonas oryzae pv. oryzae* were associated primarily with autophagy-like cell death presented by the formation of auto phagosome-like bodies in the parenchyma cells of xylem vessels. In contrary, the xa5- and xa13 mediated resistances to Xoo were found to be associated with vacuolar-mediated cell death characterized by disruption of tonoplast in the xylem parenchyma cells (Cao *et al*., 2018) [31]. The majority of R genes code for cell surface or intracellular receptors and Kourelis and van der Hoorn, (2018) [23] distinguished nine molecular mechanisms by which R proteins can elevate or initiate disease resistance that can briefly be categorized as (i). direct perception of pathogen-derived molecules on the cell surface by receptor-like proteins (RLP's) and receptor like kinases (RLKs) for example FLS2 and RLP23 in Arabidopsis, OsFLS2 in rice, FLS3, LeEIX2, SlFLS2 in tomato etc. (ii). or indirect perception of pathogen-derived molecules on the cell surface by receptor-like proteins (RLP's) and receptor like kinases (RLKs) like Cf-2 in tomato (iii). direct intracellular recognition of pathogen-derived molecules by nucleotide binding, leucine-rich repeat receptors, like L5/L6/L7 in flax or (iv). indirect intracellular recognition of pathogen-derived molecules by nucleotide binding, leucine-rich repeat receptors as exemplified by RPM1, RPS2 in arabidopsis or (v). detection via integrated domains like RGA5-A and Xa1 in rice (vi). perception of transcription activator-like effectors through activation of executor genes as executed by Xa7, Xa10 and Xa23 in rice and (vii). active loss of susceptibility for example Hm1 in maize or (viii) passive loss of susceptibility, e.g. xa5, xa13 in rice or (ix). host reprogramming mediated loss of susceptibility as shown by *mlo* gene in barley.

Applications and Future Prospects

- The rapid activation of localized defense responses at the site of pathogen infection, often associated with an HR, is the most prevalent and effective mechanism used by plants to minimize pathogen attack.
- It is possible to engineer a trigger the plat for HR by combining R and Avr gene expression in a single plant genotype,
- Gene pyramiding can serve for broad spectrum and long lasting resistance.

There are reports that the development of resistant cultivars has been the most effective and economical strategy to control diseases like bacterial leaf blight (BB) disease of rice caused by *Xanthomonas oryzae* pv. *oryzae*. Molecular markers have made it possible to identify and pyramid valuable genes of agronomic importance in resistance rice breeding. Three resistance genes (Xa4 + Xa5 + Xa21) have been incorporated by them to one genetic background which can successfully provide long lasting and broad range resistance against bacterial pathogen (Suh *et al.* 2013) [32].

Conclusion

Since the isolation, cloning and molecular characterization of the first R genes in the early 1990s, a steady amount of R genes and their interactions have been identified. Even out of yet reported 14000 NLR-encoding genes, only 191 have been shown to behave and act as R-genes (Goodstein *et al*., 2012) [33]. With the advent of novel techniques like SMRT RenSeq and other next generation sequencing-dependent techniques (Steuernagel *et al*., 2016; Witek *et al*., 2016) [34, 35], the number of annotated R genes is expected to increase in near future at a faster pace than before. Deciphering the molecular mechanisms underlying these R genes, however, will still require individual one by one examination. The underlying mechanisms of the remaining 186 R-genes out of functionally active 314 R genes are yet to be decoded. Understanding these molecular mechanisms in addition to discovering more R genes is significant to allows the transfer of these traits to other species as well as for rational disease resistance engineering, thereby encompassing the recognition spectra of R genes to outside of what is normally found in nature. For instance, engineering of PBS1 for the AvrPphB cleavage site to other pathogen-derived proteases leads to the recognition in an RPS5-dependent manner (Kim *et al*., 2016) [36]. Similarly, executor R genes have been engineered to upsurge their recognition spectra (Hummel *et al*., 2012; Zeng *et al*., 2015) [37, 38]. Rigorous studies in the past two and a half decades of R gene cloning have revealed the molecular distinctiveness of numerous R genes and disclosed the underlying mechanisms governing their functions at molecular level. However, undoubtedly much more is yet to be discovered. Understanding the structural and molecular mechanisms involved in R gene mediated resistance will allow breeders and biotechnologists to engineer resistance for the crops of the future, hence ensuring sustainable food security.

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