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Extra View

The *rpg4/Rpg5* stem rust resistance locus in barley

Resistance genes and cytoskeleton dynamics

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Two closely linked resistance genes, *rpg4* and *Rpg5*, conferring resistance to several races of *Puccinia graminis*, were cloned and characterized. The *Rpg5* gene confers resistance to an isolate of *Puccinia graminis* f. sp. *secalis* (*Pgs*), while *rpg4* confers resistance to *Puccinia graminis* f. sp. *tritici* (*Pgt*). *Rpg5* is a novel gene containing nucleotide binding site-leucine rich repeat domains in combination with a serine threonine protein kinase domain. High-resolution mapping plus allele and recombinant sequencing identified the *rpg4* gene, which encodes an actin depolymerizing factor-like protein (ADF2). Resistance against the *Pgt* races QCCJ, MCCF, TTKSK (aka Ug99) and RCRS requires both *Rpg5* and *rpg4*, while *Rpg5* alone confers resistance to *Pgs* isolate 92-MN-90. The dependency on the actin modifying protein ADF2 indicates cytoskeleton reorganization or redirection plays a role in pathogen-host interactions. *Rpg5* may interact with ADF2 to activate or deactivate its function in the resistance response. Alternatively, *Rpg5* could initiate signal transduction leading to resistance in response to detecting ADF2 protein modification. *Pgt* may redirect the actin cytoskeleton by inducing modifications of ADF2. The redirection of actin could possibly enable the pathogen to develop a haustoria-plant cell cytoskeleton interface for acquisition of nutrients.

The co-evolution of plants and pathogenic fungi has led to the development of an innate defense system in the former that activates a series of responses to resist colonization by latter. Activation of defense mechanisms begins with a receptor recognizing the threat of a potential invader. Resisting introgression at the cell periphery is the first line of defense and is dependent upon a dynamic reorganization of the challenged cell's cytoskeleton. If the initial defense strategy fails to stop penetration, a second line of defense is triggered by race specific resistance genes, resulting in a hypersensitive response (HR) and localized cell death.[1,2]

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We recently reported the cloning and characterization of two closely linked stem rust resistance genes *rpg4* and *Rpg5*.[3] The *Rpg5* gene is predicted to encode a protein with novel structure containing three *R*-gene domains: a nucleotide binding site (NBS), leucine rich repeat (LRR), and serine threonine protein kinase (STPK). The *Rpg5* predicted protein structure suggests a typical *R*-gene function in recognition of the pathogen. *Rpg5* confers resistance to an isolate of rye stem rust independent of *rpg4*, but resistance to several races of wheat stem rust requires both genes. The *rpg4* gene is predicted to encode an actin depolymerizing factor-like (ADF) protein. The dependency on the actin modifying protein ADF2 indicates cytoskeleton reorganization or redirection plays a role in the pathogen-host interaction. *Rpg5* could potentially interact with *rpg4* to activate or deactivate its function in the resistance response. Alternatively, *Rpg5* may initiate signal transduction leading to resistance in response to detecting RPG4 (ADF2) protein modification. The pathogen may modify ADF2, via effector molecules, to interfere with the resistance reaction or redirect the actin cytoskeleton. The redirection of actin could possibly enable the pathogen to develop a haustoria-plant cell cytoskeleton interface for acquisition of nutrients.

Stem Rust Resistance Genes in Barley

Wheat stem rust, caused by the pathogen *Puccinia graminis* f. sp. *tritici* (*Pgt*), was a devastating disease of wheat and barley prior to the 1950's.[4] Additionally, the rye stem rust pathogen. *P.g.* f. sp. *secalis* (*Pgs*) also can attack and cause losses in barley. In the US and Canada, durable resistance in barley was achieved through the deployment of varieties with the single resistance gene *Rpg1*, while in wheat it was achieved by pyramiding several stem rust resistance genes in one cultivar. Since the deployment of this genetic resistance in barley and wheat in the early 1940s and mid-1950s, respectively, losses due to stem rust have been minimal, and widespread stem rust epidemics were considered by many to be a thing of the past until recent appearance of *Pgt* race TTKSK (aka isolate Ug99) in Uganda in 1999.[5]

The barley *Rpg1* gene is remarkably durable and confers resistance to most races of *Pgt;* however, some races of *Pgt* and some isolates of *Pgs* are virulent on barley cultivars containing *Rpg1*. A

Pgt race with virulence for *Rpg1* (designated QCCJ) was identified on barley in the Midwestern United States in 1989.[6] *Pgt* QCCJ ultimately became one of the most common virulence types in the United States and caused local epidemics in both the US and Canada.[7] The best source of resistance to race QCCJ was discovered in the unimproved barley line Q21861.[8] Resistance in Q21861 is governed by a single gene *rpg4,* which is temperature sensitive and recessive in nature.[8,9] Genetic mapping localized *rpg4* to the long arm of barley chromosome 5H.[10]

Another resistance gene (designated *Rpg5*) conferring resistance to *Pgs* isolate 92-MN-90, also is present in line Q21861 and was mapped to the long arm of barley chromosome 5H, co-segregating with *rpg4.* Resistance to *Pgs* isolate 92-MN-90 was initially thought to be due to *rpg4*,[11] but the recent cloning of the locus has shown that *rpg4* and *Rpg5* mediated resistance is distinct.[3] The *rpg4*/*Rpg5* locus also confers resistance to *Pgt* races MCCF and RCRS.[12] *Rpg1* is generally not effective against *Pgs* isolates like 92-MN-90.

The newly emerged *Pgt* race TTKSK (aka isolate Ug99) can attack nearly 70% of the world's wheat varieties[5] and is considered a threat to global food security.[13] Moreover, race TTKSK also carries virulence for most barley varieties, including those carrying *Rpg1*.[14] Fortunately, the *rpg4*/*Rpg5* locus in line Q21861 was recently shown to confer resistance to race TTKSK (Steffenson B, unpublished).

Rpg5 Has a Novel Gene Structure Containing All Three Major *R*-Gene Domains

Innate receptors or receptor complexes act as molecular switches that, once triggered by the pathogen, set into motion signaling events resulting in two different known defense mechanisms. The first line of defense, the major component of non-host resistance and a form of basal resistance, arrests the majority of invading pathogen units at the cell periphery.[15] Pathogens that have adapted to circumvent the basal resistance encounter a second line of defense: the race specific, *R*-gene mediated resistance, associated with the HR and leading to localized cell death.

The barley *R*-gene, *Rpg5*, has a novel gene structure containing an N-terminal NBS, internal LRR, and C-terminal STPK with two predicted trans-membrane domains.[3] Many studies investigating the molecular determinants of plant disease resistance have revealed that different combinations and polymorphisms of these three distinct protein domains (NBS, LRR and STPK) comprise the vast majority of pathogen recognition receptors or components of receptor complexes for *R*-gene mediated resistance.[16] The *Rpg5* gene structure appears to be novel when compared to the Arabidopsis, Brachypodium and Oryzae genomes. PCR reactions using several *Rpg5* specific primers on genomic DNA and cDNA from several varieties of wheat and the wild wheat relative *Triticum timopheevi* identified NBS-LRR and STPK genes highly homologous to *Rpg5*, but did not detect genes or transcripts linking all three domains in a single gene (Brueggeman R, et al. unpublished). These data suggest that the *Rpg5* structure combining the NBS, LRR and STPK domains probably evolved after wheat and barley split about 10 mya[17] and may be unique to Hordeum.

The *R*-genes cloned to date have been grouped into different classes according to their protein domain structure.[16] The major class of *R*-genes possess an N-terminal NBS and a C-terminal LRR and are designated NBS-LRR *R*-genes. In relatively few cases, NBS-LRR genes have been shown to specifically interact with pathogen avirulence (*Avr*) gene products.[18-21] The guard hypothesis is an alternative to direct *R*-gene/*Avr*-gene interaction.[22] In the guard hypothesis, the *R*-genes act as surveillance proteins detecting modification of host target proteins by pathogen effectors. The direct or indirect interaction between the host *R*-gene protein and pathogen AVR protein is believed to activate the signaling cascades resulting in HR and disease resistance. These signaling cascades may involve phosphorylation via the second major class of *R*-genes, the protein kinases.

Six *R*-genes containing at least one STPK domain make up the second major *R*-gene class. The STPK *R*-genes confer resistance to bacterial and fungal pathogens and have been cloned from a wide taxa of plant species.[3,23-27] The STPK domain suggests that these proteins function in plant defense signal transduction pathways involving phosphorylation. However, STPK domain-containing genes are also known to interact directly with *Avr* genes, demonstrating a capacity to recognize non-self proteins through protein-protein interactions.[28]

Tomato *Pto*-mediated resistance against *Pseudomonas syringae* is one of the most extensively studied and understood mechanisms of disease resistance. Interestingly, *Pto* requires the presence of a second gene *Prf* (an NBS-LRR gene) for resistance.[29] Functional analysis of the *Pto*/*Prf* system indicated that specific mutations in the activation loop of the *Pto* kinase induced spontaneous symptoms similar to HR observed when plants expressing *Pto* are infected with *AvrPto* containing strains of *P. syringae*. The spontaneous HR response was *AvrPto* independent, but *Prf* dependent[30] suggesting that *Prf* acts downstream or coincident with *Pto*. It was later demonstrated that *Pto* and *Prf* do act coincidently in the signaling pathway, and that the relationship is dependent on a physical interaction between the two proteins. In this resistance complex, Pto acts as a regulatory subunit of Prf, and Prf contributes to the pathogen recognition specificity of Pto.[31] Resistance against *P. syringae* strains that carry *AvrPphB* also requires two Arabidopsis genes, RPS5, a NBS-LRR gene, and *PBS1*, a serine threonine protein kinase gene.[24] These systems indicate that resistance protein complexes require both an NBS-LRR and STPK protein for pathogen recognition and resistance.

The *Rpg5* mechanism of pathogen recognition and signaling pathway may parallel *Pto.* The *Pto*-mediated resistance requires both NBS-LRR and STPK genes for resistance, and the RPG5 predicted protein structure contains all three NBS, LRR and STPK domains with significant amino acid homology to both Pto and Prf (kinase homology to Pto is 36% aa identity and 53% aa similarity and the NBS-LRR homology to PRF is 26% aa identity and 42% aa similarity). The RPG5 protein structure suggests that the three domains act coincident with one another in barley, similar to tomato, and are required for *Rpg5* mediated resistance to *Pgs* isolate 92-MN-90. The RPG5 protein with all three domains in a single protein may facilitate the identification of other components

of the resistance complex or the pathogen AVR protein by the yeast two-hybrid assay that otherwise may be missed when using an STPK or NBS-LRR gene as bait separately. It is plausible that very few identified AVR proteins interact with their corresponding NBS-LRR *R*-genes due to incomplete interaction because the resistance complex requires both STPK and NBS-LRR proteins for complete interaction. Many of the NBS-LRR *R*-genes identified may have an STPK counterpart that has not been identified due to lack of polymorphism.

The *rpg4* Gene Encodes an Actin Depolymerizing Factor-Like Protein

Genetic mapping and allele analysis using two barley populations containing *Hv*Adf2 polymorphisms (Steptoe x Q21861 and MD2 x Q21861) identified recombinants that delimited the *rpg4* region to 1kbp. This 1kbp region contains only the *HvAdf2* gene, indicating that it is the *rpg4* gene.[3] However, the barley variety Harrington and line Sm89010, which are susceptible to *Pgt* QCCJ, contain an *rpg4* allele identical at the amino acid level to the *rpg4* allele in Q21861, which is resistant to *Pgt* QCCJ. This suggested that *HvAdf2* was not the *rpg4* gene. Further analysis of allele sequences of *rpg4* and *Rpg5* together with SNP analysis of the recombinants suggested that a functional *Rpg5* may be required in addition to *rpg4* for resistance to *Pgt* QCCJ. The *Pgt* QCCJ susceptible barleys Harrington and Sm89010 with *rpg4* alleles identical to the resistant Q21861 *rpg4* allele, do not contain a functional *Rpg5* allele. The Harrington x Q21861 recombinant #18 combines the Harrington *rpg4* allele with the Q21861 *Rpg5* gene, and it is resistant to *Pgt* QCCJ, indicating that cultivar Harrington is susceptible to this race due to a lack of a functional *Rpg5* (Brueggeman R, et al. unpublished). These data suggest that *Pgt* QCCJ resistance should behave as a single dominant gene in populations where only the *Rpg5* gene is segregating in the presence of a non-polymorphic *rpg4* gene. This hypothesis is being tested in the Harrington x Q21861 and Sm89010 x Q21861 F_1 and F_2 populations and by down-regulating the two alternative *rpg4* alleles (Brueggeman R, et al. unpublished).

Phenotype analysis of three populations (Morex, Steptoe and Robust x Q21861) revealed 1:3 segregation for resistance : susceptibility, demonstrating the recessive nature of *rpg4*.[8] The *rpg4* and *Rpg5* genes are tightly linked (~40 kbp physically) adding to the complexity of the two-gene requirement for resistance. The recessive behavior of *rpg4* implies that it encodes a non-functional ADF2 protein, resulting in resistance or that the functional *Rpg4* gene encoding a functional ADF2 protein acts as a susceptibility factor in the reaction with *Pgt* race QCCJ. The three QCCJ susceptible barley cultivars (Morex, Steptoe and Robust) have identical *Adf2* alleles at the amino acid level and differ from the resistant Q21861 allele by only three amino acids (Q39H, A101T, S135G). Three *Pgt* QCCJ susceptible progeny from the Steptoe x Q21861 population have recombinations within the *Adf2* gene and contain a functional *Rpg5* allele. These recombinant *Adf2* genes contain Q39 (Q21861-like) with T101 and G135 (Steptoe-like), showing that the amino acids at positions 101 and/or 135 are important for reaction to *Pgt* QCCJ. Since both positions involve amino acids that could be phosphorylated, i.e., either T added or S replaced, it suggests that phosphorylation may be involved in regulating ADF2 function. All *Adf2* alleles tested were expressed at the transcription level, indicating that these two amino acid polymorphisms determine susceptibility/resistance to *Pgt* QCCJ.

The Role of Actin Depolymerizing Factors in Resistance

During pathogen attack by both fungi and bacteria, the plant cytoskeleton dynamically rearranges.[2] ADF proteins, in concert with other actin binding proteins, regulate actin filament dynamics.[32] ADFs are believed to function by increasing the turnover of filamentous F-actin by depolymerization of monomeric G-actin from the pointed ends of the filaments.[33] In addition, ADFs may also nucleate the assembly of new actin filaments.[32] Many interactions between the host and pathogen may be dependent on ADF proteins, including the cytoskeletal polarization in response to non-host pathogens.[34] Cytoskeleton polarization in response to pathogen challenge is well documented;[1,2] however, very little is known concerning the signaling processes that mediate this response. Studies with the barley *mlo* non-race specific resistance system conferring resistance to *Blumeria graminis* f. sp. *hordei* using actin cytoskeleton pharmacological inhibitors and genetic interference by overexpressing a barley actin depolymerizing factor gene (*HvAdf3*), demonstrated that actin cytoskeleton function was required for basal defense against an appropriate mildew pathogen and for *mlo*-mediated non-race specific resistance at the cell wall. However, actin function was not required for several race specific immune responses. It was also demonstrated that actin cytoskeletal disruption led to increased fungal penetration by non-host pathogens.[35]

Chemical or genetic disruption of actin polymerization results in greater efficiency of non-host pathogen penetration; however, in tobacco cytoskeleton perturbation can also prime the cell for HR.[36] It was proposed that the host detects disruption of the actin cytoskeleton similar to the guard hypothesis, thus triggering the HR response. Evidence is mounting that the actin cytoskeleton is the target of plant pathogen effectors to suppress non-race specific resistance, which may signal to induce a second line of defense mediated by the pathogen specific *R*-genes.[37] There is also evidence suggesting non-host pathogens that evade the first line of defense and enter the cell are met by an HR mediated resistance that could be due to several simultaneously acting *R*-genes.[38]

ADF proteins possibly play an important role in response to pathogen challenge. It is plausible that ADF proteins are potential effector targets of fungi to circumvent a defense response. Previous studies have shown that cytoskeleton disruption was only compromised in race non-specific and non-host resistance. This begs the question: is *rpg4*-mediated resistance a form of race non-specific resistance? Our data indicate that *rpg4* functions to confer resistance to a number of different *Pgt* races, but not to all. Therefore it appears to have race specificity, but only in the presence of the typical *R*-gene *Rpg5*. In the absence of *Rpg5*, the *rpg4* gene may provide some non-host or non-race specific resistance to various pathogens.

Rpg5 and *rpg4* Interact to Confer Stem Rust Resistance

In all known *Pgt* QCCJ resistant barley varieties and recombinants, it appears that the full resistance function of *rpg4* requires the presence of a functional *Rpg5* gene. We hypothesize that *Rpg5* is the *R*-gene in this system that detects the *Pgs* and *Pgt* pathogens. *Rpg5* mediated resistance to *Pgs* isolate 92-MN-90 is independent of *rpg4*. This leads to the speculation that *Rpg5* may operate in two distinct resistance pathways, one that is similar to most race-specific *R*-gene mediated resistance mechanisms and is independent of actin reorganization events mediated by *rpg4.* The second pathway may be more like a non-race specific type of resistance mechanism dependent on *rpg4*-mediated actin reorganization. Like the Pto/Prf system, *Rpg5* may be capable of recognizing more than one *Puccina graminis* derived AVR protein, leading to differential reactions and activation of different resistance pathways.

It is plausible that *Rpg5* interacts with *rpg4* directly or indirectly to activate or deactivate the actin depolymerizing factor through phosphorylation. As discussed earlier, the resistant and susceptible alleles differ by two amino acids that are both potential phosphorylation sites. It has also been shown that ADFs can be deactivated or activated by phosphorylation or dephosphorylation, respectively, via protein kinases and phosphotases.[39,40] This activation or deactivation may lead to an incompatible or compatible reaction with pathogens. Where the actin reorganization occurs and how *R*-gene mediated resistance is dependent on this reorganization still needs to be elucidated.

An attractive hypothesis is that the invading fungus actually captures the function of the cytoskeleton to feed itself. In this case, a non-functional *adf* gene that the fungus targets may prevent the fungus from becoming established within the plant. Miklis and co-workers have suggested that although actin disruption initially enhanced pathogen entry into the cell, it may have a long-term negative effect on the compatible interaction.[1] It has been shown that in the compatible interaction between barley and powdery mildew, the haustoria (fungal feeding organs) are associated with actin filament rings, and actin filaments closely follow haustoria when invaginating the plasma membrane.[41] This is similar to what occurs in the symbiotic interaction between a mycorrhizal fungus and tobacco root cells,[42] suggesting similarity between how pathogenic and symbiotic fungi may establish the fungus-host interface for nutrient acquisition. The host cytoskeleton rearrangement machinery, initially utilized for resistance, may be redirected by the pathogen to establish a feeding mechanism that is actin filament-dependent.[1,43] It is possible that functional *Adf2* enables the fungus to produce or maintain this feeding structure, thus facilitating fungus growth or that non-functional or inappropriately functional *adf2* disrupts the actin filaments such that the fungus cannot maintain the feeding structure resulting in incompatibility.

A complete model for the function of *rpg4* and *Rpg5* in *Pgt* resistance must take into account the observations that these two genes work together and that the *rpg4* gene function is recessive and temperature sensitive. It is, of course, possible that each gene functions independently, but weakly and both are required for a strong resistance phenotype. It should be noted that both *Rpg4* alleles are expressed at the mRNA level, although we do not yet know if this is true at the protein level. The differential interaction between *Rpg4* or *rpg4* encoded proteins with *Rpg5* could be modulated by the fungus or *Rpg5* could modulate their interaction with the fungus. In order to satisfy the requirement that the *rpg4* gene action is recessive, the presence of *Rpg4* encoded ADF2 molecules must lead to susceptibility, while *rpg4* encoded ADF2 must have a neutral or negative function.

It is well documented in animal pathogenic bacteria that pathogens produce proteins that disrupt actin cytoskeleton function in order to become adapted to the host and thus become pathogenic.[44,45] There is little evidence of plant pathogenic fungi producing effector molecules that disrupt actin dynamics; however, many genera of fungi produce cytochalasins the major chemical used for pharmacological inhibition of actin filament function.[46] There is precedence of a bacterial plant pathogen secreting the effector, AvrPto, into the host cytoplasm, which disrupts the cytoskeleton polarization response. AvrPto protein has been shown to suppress callose deposition at the site of *Pseudomonas syringae* challenge, a cytoskeleton dependent response, leading to loss of basal resistance in Arabidopsis.[47] *Rpg5* could also be the guard protein monitoring the ADF2 protein for any modifications by the fungus effector; however, this would not explain why *rpg4* is recessive. *Rpg5* should still be able to guard ADF2 even when the *rpg4* gene is present in a heterozygous state.

The *rpg4*/*Rpg5* resistance system is the first link between an *R*-gene that putatively recognizes a race specific pathogen and an actin binding protein, which may be a link between the cytoskeleton dynamics occurring post pathogen interaction.

The Next Step

The molecular basis of disease resistance appears very complex, and although progress has been made in identifying many components there are still key questions unanswered. A complete signal transduction pathway leading to fungal pathogen recognition has yet to be identified and little is understood about how resistance genes activate defense signaling. It is apparent from the *R*-gene literature that different resistance pathways are activated by pathogen recognition, and there is probably cross talk and common components involved among them. We are lacking answers to some of the key questions regarding the *rpg4*/*Rpg5*-mediated mechanism of resistance that may help answer questions regarding receptor activation and dynamic cytoskeleton rearrangement. Our future research will focus on addressing some of the questions regarding how and at what level *rpg4* and *Rpg5* interact to confer resistance to stem rust.

The *rpg4*/*Rpg5* locus may answer some questions about disease resistance signaling. The novel gene structure of *Rpg5* may provide answers to why NBS-LRR and STPK proteins interact and are required components of diverse *R*-gene receptor complexes. The interaction between *rpg4* and *Rpg5*, whether direct or indirect, may also provide answers to how pathogen recognition signals the rapid rearrangement of the actin cytoskeleton and what role this rearrangement plays in the huastoria-plant cell interface. It may also be the first system where we have a pathogen effector target

that a fungus acts upon in order to perturb cytoskeleton dependent resistance.

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