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Opinion piece

When resistance gene pyramids are not durable—the role of pathogen diversity

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A major goal of plant breeding is to create cultivars that are resistant against one or more pathogens. To achieve this, breeders have traditionally introgressed resistance from existing resistant varieties or from wild crop relatives. The resulting resistant cultivar is typically overcome by an evolved pathogen population within a few years. Since the term 'durable resistance' was coined by Johnson (1979), many strategies have been proposed (e.g. Wolfe and Barrett, 1980) to achieve such long-lasting disease resistance. One proposed solution is to simultaneously 'stack' or 'pyramid' several resistance genes (*R* genes) into a single cultivar to create more durable disease resistance. Here, we consider the principles underlying *R* gene pyramids and the associated pathogen populations to predict when pyramids of *R* genes are more likely to succeed and when they are more likely to fail in providing durable resistance.

Resistance to plant pathogens can be achieved in many ways, including the manipulation of active and passive biochemical pathways associated with structural and chemical mechanisms that govern responses to pathogen infection. Genetically, one can differentiate between genes encoding quantitative effects (quantitative resistance or QR) and genes encoding qualitative major effects on disease resistance (major gene resistance or MGR) or disease susceptibility (major gene susceptibility or MGS). QR can be based on a single gene with a large effect, such as *Lr34*, or on many genes with smaller, but additive, effects that contribute to a quantitative phenotype. QR generally reduces pathogen population size compared with susceptible cultivars by decreasing infection rates or infected area, but allows some pathogen reproduction. MGS typically involves a dominant susceptibility factor that is targeted by pathogen secreted proteins (acting as effectors or toxins) and facilitates infection by necrotrophic or hemibiotrophic pathogens. MGS genes identified to date share no particular characteristics, other than that they increase levels of pathogen infection and reproduction. Most MGR genes identified to date encode a class of proteins that are nucleotide binding and contain leucine-rich repeats (NLRs). NLRs have a modular structure and act as sensors that detect pathogen-secreted effectors inside the plant cell. If these effectors are recognized by the plant, they are called avirulence (Avr) effectors. On recognition of an Avr effector, the NLR initiates a signalling cascade that typically leads to localized cell death around the pathogen ingression site. This cell death creates an incompatible environment that prevents (hemi-)biotrophic pathogens from obtaining nutrients and halts further development of disease.

From the pathogen's perspective, there are several ways to overcome or avoid detection by plant NLRs. These range from silencing of the recognized Avr effector (e.g. avoiding expression) and epigenetic changes, to insertion of transposable elements (rendering the gene non-functional) or even complete loss of Avr genes from the genome. The frequency and efficacy of the above-mentioned mechanisms are still mostly unknown. One slightly better understood mechanism is mutations in genes encoding recognized Avr effectors. The likelihood that an NLR-overcoming mutation will occur is small. Flor (1958) found in his screens that approximately three spores of 2 000 000 became virulent through natural mutations. Drake *et al.* (1998) calculated that the rates of spontaneous mutations in most organisms, including plant pathogens, typically are around 10^{-10} per base. Although many mutations will be silent or synonymous, this results in a mutation rate estimate of 10^{-5}–10^{-7} per gene, meaning that a favourable mutation (Avr to Vir) should occur once in every 100 000 to 10 million cells/spores per generation, similar to the observations of Flor (1958). The extremely large number of pathogens found in a typical field means that such favourable mutations are likely to occur regularly. For example, a sporulating powdery mildew lesion produces $\sim 10^4$ conidia per day. With 10% of the leaf area in a field infected, this results in $\sim 10^5$ lesions per square metre and thus 10^9 spores per square metre or 10^{13} spores produced per hectare per day. If the mutation rate from Avr (avirulence, with detection by the corresponding *R* gene) to avr (virulence, evasion of detection by the corresponding *R* gene) is 10^{-6}, there would, in this hypothetical example, be approximately 10^7 virulent mutant spores that can overcome an *R* gene produced in each hectare during each day under optimal conditions.

To avoid the loss of *R* gene effectiveness, multiple *R* genes can be introgressed into cultivars using a series of backcrosses, marker-assisted selection or by the creation of F1 or F2 hybrids.

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A genetic engineering approach could also be used to create artificial plasmids or to integrate constructs containing multiple NLR genes (*aka R* gene cassettes) that could be transferred into crop plants using biolistics or *Agrobacterium*. The underlying principle supporting the development of *R* gene pyramids is that a pathogen is unlikely to simultaneously mutate the series of genes encoding the corresponding Avr effectors, with the probability of simultaneous mutations decreasing as the number of NLR genes in the pyramid increases. If we take the above-mentioned mutation rate, the probability of two mutations occurring in the same pathogen strain is $10^{-6} \times 10^{-6} = 10^{-12}$, providing an output of only 10 double mutants per hectare each day in our theoretical powdery mildew population. If three NLR genes are introduced, the model predicts that only one triple mutant would be produced each day in 10 000 infected hectares. A pyramid of four *R* genes is expected to be virtually impregnable to pathogen evolution because evasion of detection would require four simultaneous mutation events in the same cell. This example illustrates well the great intellectual appeal of *R* gene pyramids, but both the *R* gene pyramids and the associated pathogens would need to simultaneously fulfil a number of criteria in order to realize the postulated benefits.

For *R* gene pyramids to be durable, the associated *R* genes should be highly effective and not leaky. This means that every strain carrying the Avr allele should be unable to infect and reproduce on the plant carrying the corresponding *R* gene. For most NLR genes affecting (hemi-)biotrophic pathogens, this appears to be the case because the hypersensitive cell death response prevents most Avr-containing spores from propagating infection on mature and healthy leaves. However, little is known about *R* gene effectiveness in immature, damaged or senescent leaves, especially for hemibiotrophs. We consider it likely that a small, but significant, amount of pathogen inoculum can survive on such leaves where the defence responses are likely to be debilitated. An *R* gene that is 99.99% effective will reduce the associated pathogen population size to 1/10 000 of that which would be expected on a susceptible cultivar. Applying this effectiveness to our hypothetical powdery mildew example, successful infection by non-mutated spores of only 0.01% of the typical leaf surface still produces a population of 10^9 spores per hectare per day, resulting in 1000 potentially virulent mutants produced per hectare each day. Because most pathogens have a generation time that is significantly shorter than that of the plant, each subsequent pathogen generation enables an exponential increase in the number of possible virulent mutants.

Second, the *R* genes should be truly novel so that the corresponding pathogen population is completely naive, leaving the corresponding Avr mutants at the very low frequencies expected under mutation–drift equilibrium. On average, $N \times \mu$ new mutants are expected to be introduced into a field population each generation, where N is the pathogen population size in the field and μ is the mutation rate. If $\mu = 10^{-6}$, approximately one virulent mutant is expected to be present amongst every million individuals (spores) in a field at mutation–drift equilibrium. If the pathogen population had already been exposed to this *R* gene in its recent evolutionary history, the frequency of the virulent mutant would be likely to be much higher as a result of selection.

Some pathogen effectors are not recognized by NLRs, but are able to suppress the host defence response or effector recognition by particular *R* genes. In addition to being novel, effective pyramids must thus consist of *R* genes that cannot be suppressed by non-targeted effectors already existing in the pathogen population. Depending on the fitness costs associated with the mutations, when avr alleles that can avoid recognition (e.g. as a result of mutations in crucial binding sites) are present at the low frequencies expected under mutation–drift equilibrium (i.e. standing genetic diversity), pyramids may fail to deliver durable resistance if the effective population size is high, e.g. if 10^{12} spores are falling every day into a field planted to a pyramid of two *R* genes. Many studies have now shown that there is substantial standing variation in Avr genes existing in natural pathogen populations, even in small sample sets. Sequence analysis of effectors from *Hyaloperonospora arabidopsidis* showed many polymorphisms in ATR13. Sequence analysis of only 16 strains, all originating from the UK, revealed 18 different alleles (Allen *et al.*, 2008). Interestingly, the N-terminal domain of the Avr protein remained conserved and mutations in the central leucine/isoleucine region had no effect on effector recognition, but domain swapping analysis revealed that non-synonymous mutations in the C-terminal part of the Avr protein affected recognition by the previously identified ATR13 receptor RPP13.

In a global collection including over 600 strains of the barley scald pathogen *Rhynchosporium commune*, more than 45% of strains had deleted the *AvrRrs1* gene *NIP1* (Schürch *et al.*, 2004). More than 20 *NIP1* alleles were found amongst ~200 sequenced strains, with most of these alleles escaping recognition by the *Rrs1 R* gene and with many different alleles found within most barley fields. Even greater diversity has been shown recently for the *AvrStb6* gene found in every tested strain of the wheat pathogen *Zymoseptoria tritici*, with more than 24 non-synonymous single nucleotide polymorphisms (SNPs) generating more than 14 amino acid variants amongst 250 global isolates, and with many different alleles found within most wheat fields (Zhong *et al.*, 2017).

In each of these cases, the virulence allele is already present in many strains in each field population, probably because the pathogen has already encountered the corresponding *R* gene in its recent evolutionary history and has evolved allelic variants that are no longer recognized by that *R* gene. These examples illustrate how standing genetic variation in the pathogen population is

often sufficient to render an *R* gene ineffective prior to its deployment into a new *R* gene pyramid.

Another prerequisite for durability is that the corresponding pathogen should only rarely recombine its genome. An *R* gene pyramid will remain effective as long as at least one component of the pyramid can recognize the corresponding Avr effector. In our hypothetical powdery mildew example, we assumed that the pathogen is strictly asexual; hence, a series of independent *de novo* mutations would be needed to defeat the *R* gene pyramid. The addition of pathogen recombination has a profound effect that is best illustrated with an extension of the powdery mildew example. Imagine three neighbouring fields planted to the same crop. The crop in field 1 contains resistance R1, field 2 is planted to resistance R2 and the third field is planted to a crop lacking any resistance. Every hectare of field 3 is releasing 10^{13} spores per day, 10^{7} of which carry avr alleles that can cause disease on the R1 and R2 crops. The incoming mutants that are not recognized by R1 are rapidly selected in the R1 field, with the result that all spores flying out of the R1 field are avrR1 mutants (i.e. mutants not recognized by the R1 NLR). The same process occurs in the R2 field, so that all spores flying out of this field are avrR2 mutants. When a plant located in field 3 is colonized simultaneously by the avrR1 mutant spores flying out of field 1 and the avrR2 mutants flying out of field 2, sexual recombination can occur. Because powdery mildew pathogens are haploid ascomycetes, if the avrR1 and avrR2 genes are unlinked, one of four of the resulting offspring will be avrR1/avrR2 and able to defeat an R1/R2 pyramid. By way of comparison, only one of 10^{12} asexual offspring flying out of any of the fields would be expected to be avrR1/avrR2 by mutation alone. Hence, a recombining pathogen population is expected to bring together independent virulence mutations much more rapidly than an asexual pathogen population. When combined with the common occurrence of mixed reproduction systems (i.e. pathogens that undergo both sexual and asexual reproduction) and a wide variety of possible asexual gene alteration mechanisms (e.g. the occurrence of accessory chromosomes and parasexual recombination in asexual lineages), it is easy to imagine how independent mutations could be recombined within a field to overcome an *R* gene pyramid.

In addition to low recombination rates, the corresponding pathogen should exhibit a low level of gene flow amongst field populations. High gene flow amongst field populations increases the overall pathogen population size, leading to higher pathogen genetic diversity and an increased likelihood of beneficial recombination events. High gene flow has been observed in many pathosystems. Particularly problematic are cases in which there is significant gene flow between pathogen populations found on domesticated hosts and their wild relatives, especially when the pyramided resistance genes originate from the wild crop relatives. An excellent example of gene flow between wild and domesticated populations occurs every year in the Durance Valley in France (Xhaard *et al.*, 2012). The rust fungus *Melampsora larici-populina* spreads among poplar trees growing along the Durance River each spring after overwintering on larch trees. In spring, inoculum can first be observed on cultivated poplar stands, but, shortly after, it is also detectable in wild stands; this difference is most likely a result of ecological conditions (high density monocultures) that favour the earlier development of disease in the cultivated stands. Genetic analysis showed that the wild and cultivated pathogen populations belonged to genetically differentiated populations that were ultimately derived from the same wild population after strong selection in the cultivated populations as a result of the presence of the R7 rust resistance gene. Indeed, most cultivated populations contained the unrecognized avr7 allele, whereas wild populations carried the recognized Avr7 allele. Hybrids between the populations were rare, presumably because of the strictly asexual reproduction during epidemics on poplar; however, a small number of mixed genotypes and even hybrids were observed in the wild stands close to the larch populations in which sexual reproduction takes place.

One consistent theme that emerges across all of these examples is the need to keep the pathogen effective population size as low as possible in order to decrease the amount of standing genetic variation in the pathogen population and increase the effectiveness of the *R* gene pyramid. For example, the adoption of reduced tillage practices to preserve soil leads to an increase in the amount of crop debris remaining on the soil surface, opening a niche for some pathogens to survive and even increase their effective population size between growing seasons. This may, in part, explain the growing global importance of the wheat pathogen *Z. tritici* and other necrotrophic pathogens that are good saprophytes. Many management practices can diminish pathogen population sizes, including regular crop rotations, planting a greater diversity of crops over smaller spatial scales, improved stubble management and targeted fungicide applications. QR typically lowers pathogen population size by limiting pathogen reproduction and also tends to be non-selective, enabling the persistence of Avr alleles in the affected pathogen population. By way of comparison, MGR typically removes all strains carrying the Avr allele and rapidly drives the avr mutants to a high frequency. When the avr mutants have no specific evolutionary advantage, or exhibit avr-related fitness costs, the virulent mutants will remain at a low frequency. Hence, QR can be used to protect the *R* genes deployed in a pyramid and extend their life expectancy. The effectiveness of *R* gene pyramids is likely to improve considerably when they are deployed in combination with QR or other management practices that reduce the pathogen effective population size.

The development of *R* gene pyramids can take a considerable amount of time, especially if they are based on transgenic or

cisgenic *R* gene cassettes that require government approval prior to field deployment. Given that several of the assumptions associated with the theory supporting the deployment of *R* gene pyramids are violated in many cases, we argue that the use of *R* gene pyramids should be reconsidered until breeders and pathologists have acquired a deep enough understanding of the pathogen in question, including its population structure, reproductive strategy and sufficient knowledge of all factors that will affect the diversity and 'evolvability' of the corresponding Avr effectors in the pathogen population. Adequate knowledge of the pathogen's population biology, including its effective population size and the amount of standing variation existing in the current populations on wild and cultivated hosts, will enable a rough calculation of the likelihood that an *R* gene pyramid will be overcome. Careful analyses of standing genetic variation will greatly reduce the chances of wasting effort on deploying *R* genes that have already been broken.

A better understanding of pathogen Avr diversity and how it evolves might allow us to engineer more complex *R* gene deployment strategies which more closely resemble those that evolved in wild plant populations that rarely experience epidemics. Wild plant populations typically maintain many allelic variants for at least some *R* genes in their genome. In the highly inbred *Arabidopsis thaliana*, populations maintain a polymorphism for the RPP13 R protein that recognizes the ATR13 effector, with at least 49 alleles observed in UK metapopulations. These alleles show varying degrees of recognition of ATR13, ranging from no to full recognition. Notably, six of 16 ATR13 alleles were not recognized by any of the RPP13 alleles tested (Hall *et al.*, 2009). Similarly, in the inbreeding wild tomato species *Solanum pennellii*, there is strong evidence that heterozygosity is maintained for a small number of *R* genes (Stam *et al.*, 2016). Amongst them is a tomato homologue of RPP13, suggesting that the maintenance of multiple alleles within the population is crucial for the effectiveness of certain essential *R* genes.

Although most agricultural fields are based on homogeneous, high-density monocultures, it is easy to imagine how cloning and gene editing techniques could be used to create *R* gene diversity within agricultural fields that could mimic the *R* gene diversity found in natural wild populations. Experiments using cultivar mixtures composed of closely related cultivars carrying different *R* genes have shown that increasing diversity within agricultural fields provides an effective way to maintain disease resistance in crops (Mundt, 2002). Mark–release–recapture experiments with three cereal pathogens have provided evidence that increasing host genetic diversity by growing cultivar mixtures reduces effective population size and imposes diversifying selection, resulting in a reduced rate of evolution for each pathogen population on their respective hosts (Zhan and McDonald, 2013).

We believe that reliance solely on the deployment of an *R* gene pyramid is unlikely to provide durable control for many pathogens, especially those with large populations, mixed reproductive systems and substantial gene flow. Although the deployment of mixtures of major *R* genes within a field and/or greater deployment of QR offer other promising strategies to extend the useful lifespan of *R* genes, all control strategies based on *R* genes are likely to last even longer if they are combined with other management practices, such as crop rotations and judicious applications of fungicides, which lower the pathogen effective population size. We conclude that a crucial concept to consider when aiming for durable resistance is: know your enemy, and its diversity.

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