Plant Biotechnology Journal
Open Access

Plant Biotechnology Journal (2023) **21**, pp. 1938–1951 doi: 10.1111/pbi.14106

Review Article

Resistance that stacks up: engineering rust and mildew disease control in the cereal crops wheat and barley

Peter M. Dracatos[1,*], Jing Lu[2,3,4,5], Javier Sánchez-Martín[6] and Brande B.H. Wulff[2,3]

[1]*La Trobe Institute for Sustainable Agriculture & Food (LISAF), Department of Animal, Plant and Soil Sciences, La Trobe University, VIC 3086, Australia*
[2]*Plant Science Program, Biological and Environmental Science and Engineering Division (BESE), King Abdullah University of Science and Technology (KAUST), Thuwal, Saudi Arabia*
[3]*Center for Desert Agriculture, KAUST, Thuwal, Saudi Arabia*
[4]*College of Life Sciences, Sichuan University, Chengdu, China*
[5]*Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, China*
[6]*Department of Microbiology and Genetics, Spanish-Portuguese Agricultural Research Center (CIALE), University of Salamanca, Salamanca, Spain*

Received 2 January 2023;
revised 5 June 2023;
accepted 9 June 2023.
**Correspondence* (email p.dracatos@latrobe.edu.au)

Keywords: biotechnology, biotrophic pathogens, cereal rusts, genomics, powdery mildew, resistance.

Summary

Staying ahead of the arms race against rust and mildew diseases in cereal crops is essential to maintain and preserve food security. The methodological challenges associated with conventional resistance breeding are major bottlenecks for deploying resistance (*R*) genes in high-yielding crop varieties. Advancements in our knowledge of plant genomes, structural mechanisms, innovations in bioinformatics, and improved plant transformation techniques have alleviated this bottleneck by permitting rapid gene isolation, functional studies, directed engineering of synthetic resistance and precise genome manipulation in elite crop cultivars. Most cloned cereal *R* genes encode canonical immune receptors which, on their own, are prone to being overcome through selection for resistance-evading pathogenic strains. However, the increasingly large repertoire of cloned *R* genes permits multi-gene stacking that, in principle, should provide longer-lasting resistance. This review discusses how these genomics-enabled developments are leading to new breeding and biotechnological opportunities to achieve durable rust and powdery mildew control in cereals.

Introduction

Whole-grain cereals within the Triticeae including wheat (*Triticum* sp.), barley (*Hordeum vulgare*), rye (*Secale cereale*) and triticale (× *Triticosecale* Wittmack) are rich sources of calories, essential vitamins, minerals and phytochemicals that both nourish and protect humans and animals from ailments such as heart attack and cancer (Aune *et al.*, 2016). Of these, wheat is the most widely consumed globally, while barley is the world's fourth most important cereal, used primarily in malt production for alcoholic beverages and as grain feed for livestock and human food (Figure 1; Tables S1 and S2). Although cereal cultivation is widespread, global demand and the ability to reliably cultivate large quantities of cereal grain varies substantially between wheat and barley and within different regions of the world (Figure 1).

Wheat and barley are hosts for a myriad of pests and diseases that decrease projected yield and threaten crop production targets (Oerke and Dehne, 2004; Savary *et al.*, 2019). For example, approximately 20% of global wheat production is lost to infection by pests and diseases (Figure 1a). Monoculture and climate change favour the emergence of new, highly virulent pathogen variants that reduce yield, posing a serious threat to global food security (Velásquez *et al.*, 2018). Historically, foliar diseases of cereals have been controlled by (i) eradicating alternate hosts (Barnes *et al.*, 2020), (ii) breeding resistant cvs. (Hafeez *et al.*, 2021) and, more recently, (iii) spraying with systemic fungicides (Deising *et al.*, 2008). Fungicides create selection pressure that encourages the emergence of fungicide-resistant crop pathogen variants (Tucker *et al.*, 2015) as well as environmentally ubiquitous yeast and *Aspergillus* variants which can transfer to humans to cause infections resistant to medical antifungals (Castelo-Branco *et al.*, 2021; Gow *et al.*, 2022; Rhodes *et al.*, 2022). Fungicides are also expensive, harmful to the environment and have been reported to deplete soil populations of mycorrhizal fungi, with negative consequences for crop mineral nutrition (Edlinger *et al.*, 2022). Finally, exposure to fungicides has been linked to human cancers and other health disorders (Alhanti *et al.*, 2022; Hutter *et al.*, 2021; Lerro *et al.*, 2021). Although genetic resistance has been used to control crop diseases, for example against powdery mildew in wheat and barley (Brown and Wulff, 2022), the durability of the resistance is often limited due to the ability of the pathogen to overcome major race-specific resistance (*R*) genes (McDonald and Linde, 2002).

Fungal pathogens that cause disease in plants are categorized based on their feeding mechanisms. Biotrophic fungi colonize living plant cells and require carbon and nutrients provided by the host for growth and sporulation (Lorrain *et al.*, 2019). Biotrophic fungal pathogens have a complex biochemical and structural relationship with their hosts (Dracatos *et al.*, 2018). They contain specialized structures, called haustoria, that evaginate the host plasma membrane both creating a nutrient sink and suppressing host defences through the secretion of effector proteins (Lorrain *et al.*, 2019). Often perceived to lack sophistication relative to

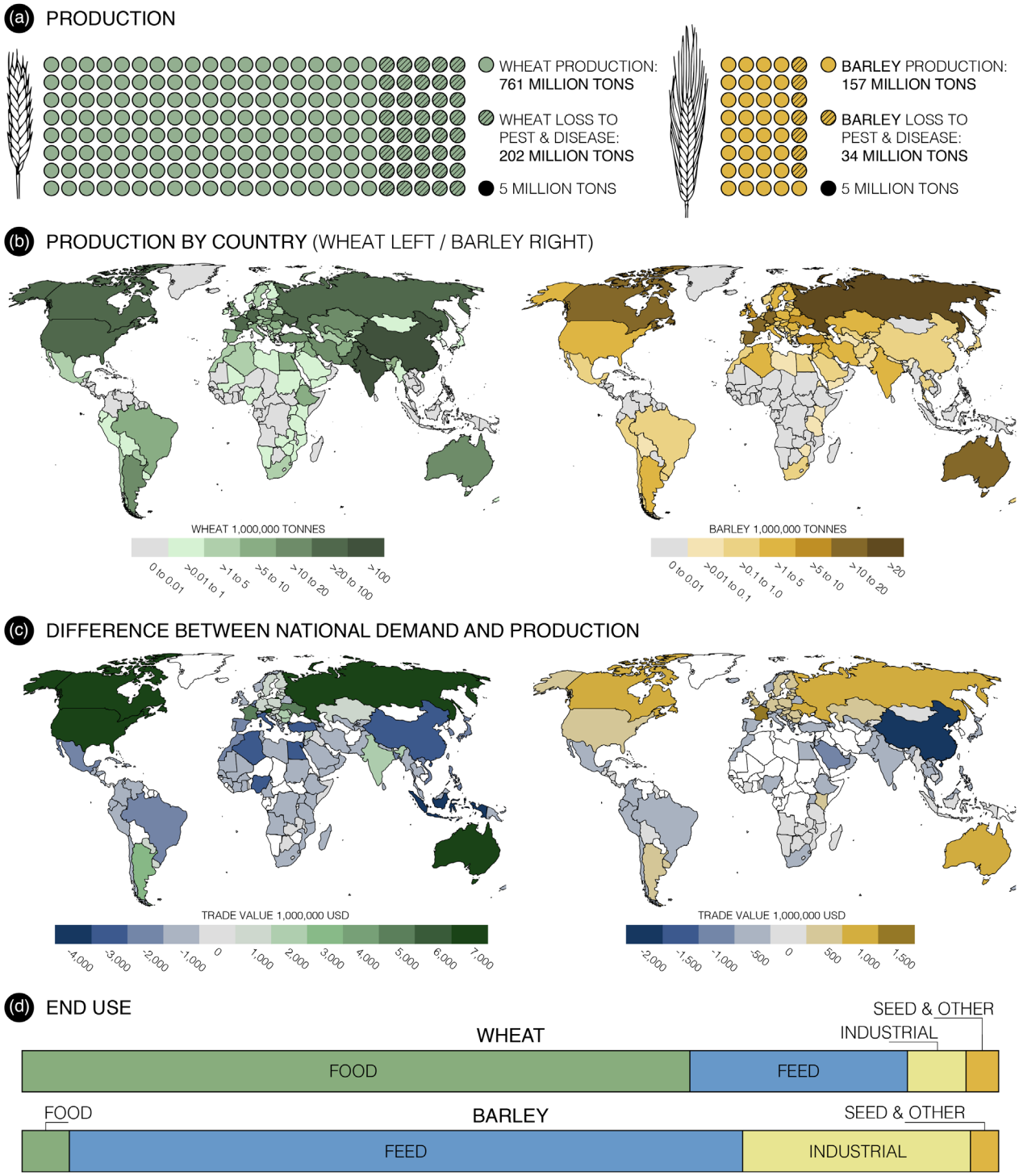

Figure 1 Global wheat and barley production relative to demand and the effect of pests and diseases on grain yield and end-use data. (a) Estimates of yield loss to pests and diseases adapted from expert-based assessments of crop health (Oerke and Dehne, 2004; Savary *et al.*, 2019). (b) Worldwide wheat and barley production by country in 2017 (FAO stats accessed on 1 September 2022). (c) Trade deficit/surplus calculated as the value in US dollars between national demand and production for wheat and barley. (d) End-use data for wheat and barley in 2020/21 as a proportion of whole production. https://www.igc.int/en/markets/marketinfo-sd.aspx (accessed on 1 September 2022). For the purpose of this map, the authors used production and trade data reported by the FAO and the World Bank. The authors remain neutral on issues of disputed regions and borders.

their biotrophic counterparts, many necrotrophic fungal pathogens of cereals hijack host resistance responses deployed against biotrophs by targeting *R* genes and receptor kinases commonly associated with incompatible reactions in biotrophic interactions (Faris and Friesen, 2020; Hammond-Kosack and Rudd, 2008). Therefore, rather than relying on haustoria, necrotrophic fungi

tend to overpower their hosts with a cocktail of virulence factors and secreted molecules such as lytic enzymes (Koeck *et al.*, 2011; Voegele and Mendgen, 2003). This often-neglected perspective has important implications for the utility and pyramiding of *R* genes in breeding programmes and the development of gene stacks using biotechnological approaches.

The initial recognition of non-self or pathogen-associated molecular patterns (PAMPs) and subsequent mounting of downstream defence responses by plants is initiated at the cell surface by membrane-associated pathogen recognition receptors (PRRs) and are collectively referred to as PAMP-triggered immunity (PTI; Jones and Dangl, 2006). Adapted biotrophic pathogens have co-evolved with their hosts and consequently produce effectors that enable them to evade PTI, colonize surrounding cells, and cause disease. The multi-layered immune system from the host plant comprises large gene families encoding membrane-bound and intracellular receptor proteins that recognize race-specific pathogen effector molecules, leading to effector-triggered immunity (ETI) (Dodds and Rathjen, 2010; Jones and Dangl, 2006). Recent studies in Arabidopsis have demonstrated that ETI and PTI potentiate each other (Ngou *et al.*, 2021; Yuan *et al.*, 2021) augmenting the importance of ETI gene-based stacks. Our understanding of the genes involved in host-biotrophic pathogen interactions is advanced, especially for membrane receptors that mount PTI and common *R* genes encoding nucleotide-binding leucine-rich repeat (NLR) proteins that trigger ETI. The products of other non-canonical *R* genes underscore a level of mechanistic diversification that is less well understood (Sánchez-Martín and Keller, 2021); however, in combination with NLRs, they are likely to provide a formidable barrier for pathogens to overcome (Brun *et al.*, 2010).

New genomic approaches have enhanced the rate of gene isolation, improving our understanding of the molecular basis of host disease resistance and acquisition of virulence by the pathogen. In the case of biotrophic pathogens that infect cereals within the Triticeae, this molecular insight has been facilitated by an in-depth knowledge of host–pathogen genetics for each of the respective pathosystems. Cereal rust (*Puccinia* spp.) and mildew (*Blumeria* spp.) pathogens are highly host-specific, having co-evolved with a narrow range of host species (Dracatos *et al.*, 2018). Among *Puccinia* species, those that attack wheat are the most economically important (Savary *et al.*, 2019). Stem rust, an epiphytotic disease caused by *P. graminis* f. sp. *tritici* (*Pgt*), can lead to crop failure during heavy epidemics, while stripe rust, caused by *P. striiformis* f. sp. *tritici* (*Pst*), and leaf rust, caused by *P. triticina* (*Pt*), cause losses of up to 50% on susceptible varieties. For the mildew fungus *B. graminis*, the pathogenic strains adapted to wheat (f. sp. *tritici*), or barley (f. sp. *hordei*) cause the most economic losses (Conner *et al.*, 2003; Everts *et al.*, 2001). In this review, we discuss recent developments in the molecular genetic analysis of wheat and barley interactions with rust- and powdery mildew-causing fungi and explore opportunities to engineer durable host resistance.

Capturing functional diversity through genomics-enhanced association genetics

The availability of reference genomes and the development of gene-class-specific capture arrays have undoubtedly enhanced *R* gene cloning efforts using map-based cloning (Klymiuk *et al.*, 2015; Walkowiak *et al.*, 2020), mutational genomics (Sánchez-Martín *et al.*, 2016; Steuernagel *et al.*, 2016) and genome-wide association studies (GWAS) (Arora *et al.*, 2019). Moreover, unbiased complexity reduction approaches, such as TACCA (targeted chromosome-based cloning via long-range assembly) or MutChromSeq, that are based on chromosome sorting and sequencing of wild-type plants and chemically induced mutants have been applied to isolating genes involved in disease resistance (Dracatos *et al.*, 2019; Sánchez-Martín *et al.*, 2016; Thind *et al.*, 2017; Yu *et al.*, 2022) and morphological characteristics (Ford *et al.*, 2018) in cereals. A highly efficient approach combining PacBio Iso-Seq of the wild-type parental genotype with RNA sequencing of multiple mutants was used to clone the wheat leaf rust *R* gene *Lr9* (Wang *et al.*, 2023). This approach substantially decreased the cost and time of gene cloning, mitigating the necessity to sort chromosomes and is highly amenable for non-reference wheat *R* gene donor lines. The necessity for genome complexity reduction methodology is decreasing as researchers capitalize more on the rapidly declining costs of DNA sequencing to generate reference genomes of resistant accessions (Athiyannan *et al.*, 2022; Li *et al.*, 2022; Yu *et al.*, 2022) or sequence genetically diverse panels for GWAS (Gaurav *et al.*, 2022).

Mining germplasm collections for traits of interest and introducing them into crop breeding programmes is far from novel. Fundamental to this activity are the tens of thousands of crop accessions maintained in global gene banks along with passport data (a basic description of the accession such as accession name, genus, country of origin, acquisition date, etc.). However, errors in labelling can lead to duplications (Singh *et al.*, 2019). Milner *et al.* (2019) generated molecular passport data by performing genotyping-by-sequencing (GBS) on the entire German *ex situ* gene bank of more than 23 000 accessions of wild and domesticated barley. This resource provided insight into the global population structure of domesticated barley and, through increased marker density and population size, greater analytical power for GWAS. Furthermore, phenotyping diverse subsets of the barley gene bank identified marker–trait associations underlying previously cloned genes controlling virus resistance and morphological traits (Milner *et al.*, 2019). In a separate study, whole-genome shotgun sequencing of 242 diverse Tausch's goatgrass (*Aegilops tauschii*) accessions revealed that the D genome of wheat was sourced from geographically distinct lineages, while GWAS identified and validated candidate genes underlying stem rust (*SrTA1662*) and powdery mildew (*WTK4*, [*WHEAT TANDEM PROTEIN KINASE4*]) *R* genes (Gaurav *et al.*, 2022). These approaches facilitate rapid identification of previously uncharacterized alleles for resistance to biotrophic fungal pathogens in cereal crops.

Pan-genomics reveal the extent of variation at *R* loci

Molecular passport data can be used to select representative genotypes suitable for sequencing and assembling chromosome-scale reference genomes. Pan-genome projects in bread wheat (*Triticum aestivum*) (Walkowiak *et al.*, 2020) and barley (Jayakodi *et al.*, 2020) have unravelled the extent of intraspecies structural variation on both micro and macro scales. The availability of a pan-genome addresses the most common bottleneck in gene cloning projects, which is over-reliance on gene order and representation in a single available reference genome. In the

case of the barley pan-genome, representative barley genotypes were selected from each of the major global gene pools based on the GBS data from 23 000 barley accessions reported in Milner *et al*. (2019). Further selection criteria were imposed to maximize geographical diversity including row type and growth habit. The utility of the barley and wheat pan-genomes lies in being able to use the information from the most closely related genotype or directly from the sequenced genotype harbouring the trait of interest as a reference genome, thus limiting the number of single nucleotide polymorphisms (SNPs), presence/absence variations (PAVs) and inversions.

Gene duplication and diversifying selection have given rise to a clustered distribution of NLRs in plant genomes (Lee and Chae, 2020). Pan-genome variants, such as PAVs, are especially relevant for NLRs and other types of *R* genes. Often, the NLR conditioning the resistance is absent or has reduced functionality in susceptible accessions, for example due to a premature stop codon (Sánchez-Martín *et al*., 2016). A bread wheat pan-genome study illustrated this point when examining NLR gene expansion in the genome assemblies of eight diverse elite wheat cvs., the Chinese Spring landrace and spelt wheat (*Triticum spelta*; Walkowiak *et al*., 2020). A *de novo* annotation of their NLR complements identified, on average, 2500 full-length NLR loci per accession. Only 31%–34% of the NLR signatures were shared across all 10 genomes and the number of unique NLRs ranged from 22 to 192 per genome, suggesting that incorporating GM stacks of *R* genes should not be cause for concern. The total NLR complement of all 10 wheat lines consisted of 5905 (98% identity) to 7780 (100% identity) unique NLRs. Furthermore, the authors estimated that 90% of the NLR complement was reached at between eight (considering 95% sequence identity) and 11 wheat lines (considering 100% protein sequence identity). These findings highlight the size and complexity of the wheat immune receptor complement, the extent of PAVs between accessions, and the dynamic evolution of the plant immune system (Walkowiak *et al*., 2020).

The releases of the wheat and barley pan-genomes have already been and will continue to be important for isolating non-canonical *R* genes. For example, two recent studies utilized sequenced chromosome-scale assemblies from the wheat and barley pan-genomes to clone the leaf rust *R* genes *Leaf rust 14a* (*Lr14a*) (Kolodziej *et al*., 2021) and *Resistance to Puccinia hordei 3* (*Rph3*) (Dinh *et al*., 2022) from wheat and barley, respectively. Most recently the resistance gene *Rph7* was cloned using comparative sequence and expression analysis utilizing the updated barley pan-genome (Chen *et al*., 2022). *Lr14a*, *Rph3* and *Rph7* encode an ankyrin membrane-bound repeat protein, putative executor protein and NAC transcription factor, respectively, which are race-specific non-canonical resistance proteins. In the case of *Rph3*, identification of a large 80-kb deletion in the resistant barley cv. Barke relative to the reference genome assembly of the susceptible cv. Morex delimited the candidate locus to two possible genes (Dinh *et al*., 2022). Similarly, cloning and characterization of *R* genes encoding kinase fusion proteins, such as *Resistance to Puccinia graminis 1* (*Rpg1*) (Brueggeman *et al*., 2002), *Yellow rust resistance 15* (*Yr15*) (Klymiuk *et al*., 2015), *Lr9* (Wang *et al*., 2023), *Stem rust resistance 43* (*Sr43*) (Yu *et al*., 2023), *Sr60* (Chen *et al*., 2020), *Sr62* (Yu *et al*., 2022), *WTK4* (Gaurav *et al*., 2022), *Powdery mildew resistance 4* (*Pm4*) (Sánchez-Martín *et al*., 2021), *Pm24* (Lu *et al*., 2020), *Pm13* (Li *et al*., 2023), *Pm57* (Liu *et al*., 2023) and *Rwt4* (synonym of *Resistance to Magnaporthe grisea 1*; *Rmg1*) (Arora *et al*., 2023), along with the Kolodziej *et al*. (2021) and Dinh *et al*. (2022) studies suggest that as many as 35% of major, dominant, race-specific *R* genes to biotrophic pathogens of wheat and barley do not encode canonical NLR immune receptors (Table S3). Many of these (18 out of 85, 20%) encode protein kinase proteins containing fusion domains. Despite the well-documented involvement of protein kinases as resistance proteins across the plant kingdom, those including fusion domains have to date only been reported within the Triticeae (Fahima and Coaker, 2023). It has been postulated that the large and, in the case of wheat, polyploid genomes might potentiate this genomic innovation through *de novo* gene duplication and fusion events (Sánchez-Martín and Keller, 2021).

Improvements to long-read sequencing technologies continue to enhance our ability to generate ultra-contiguous chromosome-scale assemblies, thus further improving the efficacy of *R* gene identification. Athiyannan *et al*. (2022) combined PacBio high-fidelity long reads, optical mapping and chromosome conformation capture (HiC) to generate an improved 14.7-Gb chromosome-scale assembly of the South African stripe rust-resistant bread wheat cv. Kariega. These authors improved the N_{50} contig length previously reported for both short-read and PacBio long-read sequencing technologies by 150- to 600-fold, to 30 Mb. The improved genome assembly permitted efficient isolation of the race-specific *R* gene *Yr27*. Taken together, these wheat and barley pan-genome studies highlight opportunities to accelerate the cloning and characterization of *R* genes. To maximize the potential of pan-genomes and sequenced germplasm diversity panels, it will be critical to determine their *R* gene complements by performing detailed multi-pathotype testing using diverse rust and mildew isolates with contrasting pathogenicity profiles. This comprehensive analysis will help determine the presence of previously identified genes, allowing promising accessions to be shortlisted for preliminary genetic analysis to map novel *R* loci.

NLR-integrated domains reveal the diversity of pathogenicity targets in ETI

NLRs are immune receptor proteins that typically contain three domains. The central nucleotide-binding domain is common to all NLRs. Most NLRs also have a C-terminal leucine-rich repeat (LRR) domain, which confers pathogen recognition specificity, and an N-terminal interaction domain involved in signalling and subsequent cell death (Duxbury *et al*., 2020). In monocots (all cereal species), the N terminus typically contains a coiled coil (CC) domain. By contrast, dicots have either a CC domain or a toll interleukin receptor (TIR) domain. Recent NLR gene cloning and functional studies have unravelled a diverse array of atypical-integrated domains (IDs) that act as decoys for pathogen effectors and trigger a specific immune reaction when interacting with the decoy (Marchal *et al*., 2022). Examples include zinc-finger (ZF), BED, and WRKY domains of transcription factors, which are common targets for effector proteins as they play a crucial role in regulating signal transduction during basal plant immunity (Sarris *et al*., 2015). In some cases, NLR genes encode immune receptor proteins with ID combinations that are species-specific or are common across different cereal and crop species. Map-based cloning of the stripe rust *R* gene *YrU1* from red wild einkorn wheat (*Triticum urartu*) revealed that it encodes a CC-NBS-LRR protein with an N-terminal ankyrin-repeat (ANK) domain and a C-terminal WRKY domain (ANK-NLR-WRKY). Further empirical

searches determined that this unusual NLR protein structure is only found in *Triticum* species (Wang *et al*., 2020).

The ZF-BED domain was originally characterized in the fruit fly (*Drosophila melanogaster*) through mutagenesis studies and was subsequently shown to be essential for *Xa1*-mediated resistance to bacterial blight in rice (Yoshimura *et al*., 1998). In bread wheat, two BED domain-containing NLRs (*Yr5* and Yr7), encoded by a complex NLR gene cluster with unique pathogen specificity, confer resistance to stripe rust (Marchal *et al*., 2018). The discovery that the barley leaf rust *R* gene *Rph15* also encodes an NLR-BED domain-containing protein suggests that different rust pathogen species that are adapted to distinct hosts may have effectors targeting similar transcription factor domains (Chen *et al*., 2021). Functional studies in other plant species demonstrated that the binding of effectors with IDs can activate plant defence. The WRKY domain of RESISTANT TO RALSTONIA SOLANACEARUM 1 (RRS1), an NLR from Arabidopsis (*Arabidopsis thaliana*), is an integrated decoy that is targeted by two effector proteins from different pathogens whose binding activates RRS1-mediated resistance (Sarris *et al*., 2015). Analogously, the NLR proteins RGA5 and Pik-1 from rice contain heavy metal-associated (HMA) domains that act as a decoy for effectors of the rice blast pathogen *Magnaporthe oryzae* (Guo *et al*., 2018). De la Concepcion *et al*. (2021) engineered a variant of Pik-1 (Pik1h) with a single amino acid substitution in the HMA domain that broadens its recognition specificity to corresponding Avr-Pik effectors. Increasing our understanding of the diversity of IDs within cereal NLR proteins will broaden our toolkit for engineering resistance to biotrophic pathogens such as rusts and mildews.

Diverse 'gatekeepers' of host specialization

Proteins encoded by structurally distinct *R* gene classes have key roles in forming host specificity barriers. A pertinent example is in wheat, where the two *R* genes *Rwt3* and *Rwt4*, encoding an NLR and a tandem kinase, respectively, act as host specificity barriers against non-*Triticum* blast pathotypes (Arora *et al*., 2023). In *Hordeum* spp., orthologous cell wall-associated receptor kinase genes from cultivated (*H. vulgare*) and bulbous (*H. bulbosum*) barley conferred both partial and non-host resistance to adapted and non-adapted leaf rust pathogens, respectively (Wang *et al*., 2019). The partial resistance quantitative trait locus (QTL) *Rphq2* (*Resistance to P. hordei 2*) from Dutch barley cv. Vada confers quantitative resistance to adapted strains of the leaf rust pathogen *P. hordei* and non-host resistance to leaf rust strains adapted to bulbous barley. In parallel, *Rph22* from *H. bulbosum*, which maps to the same physical position as *Rphq2*, confers partial resistance to *P. bulbosii* and non-host resistance to *P. hordei*. Positional cloning and phenotypic validation of each orthologous kinase in a susceptible transgenic background determined that *Rphq2* and *Rph22* both conferred a stronger non-host resistance response to heterologous leaf rust pathogens relative to the partial response to their respective adapted leaf rust pathogens (Wang *et al*., 2019). The authors subsequently hypothesized that leaf rust isolates adapted to cultivated (*P. hordei*) and bulbous (*P. bulbosii*) barley had co-evolved with their respective hosts to mitigate perception by host receptors by lowering ligand recognition.

Shared genetic architecture of *R* loci within and between closely related cereal genomes is often characterized by diverse pathogen specificity. The *Mla* (*mildew resistance locus a*) clade on the short arm of chromosome 1H in barley is exemplified by its pronounced allelic diversification (Jørgensen, 1994). Recent phylogenetic analysis of the wheat stem rust R proteins Sr33 and Sr50 showed them to be orthologs of barley Mla proteins based on amino acid similarity to Mla1, Mla6 and Mla9, and shared synteny between the group 1 chromosomes within the Triticeae (Dracatos *et al*., 2019). This observation suggests that, although evolving from a common ancestor, NLRs from the Mla clade have evolved distinct resistance specificities to different biotrophic fungi both within and between respective crop species. In some instances, however, NLRs can have dual-pathogen recognition specificities conferring race-specific resistance to adapted pathogens and resistance to pathogen variants adapted to closely related host species. For instance, a 522-bp region of the sequence encoding the LRR domain within *Mla8* determines dual specificity for recognizing barley powdery mildew and wheat stripe rust (Bettgenhaeuser *et al*., 2021). *Mla8* represents a striking example of the shared genetic architecture within cereal genomes for resistance to both adapted and non-adapted biotrophic pathogens of different genera, implicating this *R* gene in host species specificity to the wheat stripe rust pathogen. Barley is a host to adapted *P. striiformis* strains; however, *Mla8* did not confer resistance to the *P. striiformis* f. sp. *hordei* isolates tested. These two studies emphasize the importance of further molecular characterization and understanding to be able to manipulate non-host resistance traits in cereal crops for biotechnological applications. In parallel, understanding the molecular signatures of host specificity between biotrophic pathogen isolates specialized to closely related host cereal grasses (formae speciales) using pathogenomics may help predict future host jumps.

Utilizing cloned recognized effector genes to confirm function and predict NLR gene durability

Cloning the pathogen effectors corresponding to broadly effective individual race-specific *R* genes plays an important role in determining *R* gene function. Compared to the 85 *R* genes cloned from *Triticum* and *Hordeum* spp. (including 49 NLRs), only 22 corresponding recognized effector genes encoding secreted pathogen effectors have been cloned (Tables S3 and S4). Effector discovery has lagged that of *R* gene cloning due to the challenges of working with obligate biotrophic fungal pathogens and due to the unexpected genetic complexity controlling avirulence in some cases, as attested by the recent discovery of two pathogen loci governing virulence to wheat resistance gene *Pm1a* (Hewitt *et al*., 2021; Kloppe *et al*., 2023). However, going forward, the large number of cloned *R* genes combined with recent progress in developing transient expression systems (Lorrain *et al*., 2019) and the development of gain-of-virulence mutant libraries towards targeted *R* genes, as recently applied to wheat rusts (Kangara *et al*., 2020; Upadhyaya *et al*., 2021), will facilitate effector biology studies by permitting rapid functional assays to study their specificity. Avr gene cloning studies could also be enhanced and/or more efficient through improved *in silico* rust effector predictor tools to reduce the number of candidate effectors for functional validation. For example, artificial intelligence-guided computational structural genomics has led to more accurate and better prediction of candidate pathogen effector repertoires (Seong and Krasileva, 2021, 2023). Combining this with high-throughput transient expression assays in protoplasts will likely increase the rate of Avr-effector discovery (Arndell *et al*., 2023; Wilson *et al*., 2023).

Other major drivers include recent advancements in sequencing technologies, such as PacBio SMRT sequencing and chromosome conformation capture sequencing (Hi-C), which have enabled haplo-phasing of dikaryotic rust genomes to increase assembly quality (Duan *et al*., 2022; Schwessinger *et al*., 2018). Numerous recognized effector genes were previously cloned in the flax rust (*Melampsora lini*) experimental system; however, to date, only three *Avr* genes have been characterized from the wheat stem rust pathogen *Pgt*: *AvrSr35* (Salcedo *et al*., 2017), *AvrSr50* (Chen *et al*., 2017), and most recently *AvrSr27* (Upadhyaya *et al*., 2021).

One mechanism contributing to the breakdown of rust resistance through loss of avirulence in pathogen populations is the proliferation and mobilization of transposable elements within rust pathogen genomes. For instance, transposon-mediated disruption of *AvrSr35* resulted in natural *Sr35*-virulent *Pgt* isolates (Salcedo *et al*., 2017). Loss of avirulence can also occur via large-scale chromosomal changes in pathogen genomes. For example, in the case of *AvrSr50*, a spontaneous deletion-mutant was identified, which allowed the identification of *AvrSr50* by comparative sequence analysis (Chen *et al*., 2017). The crystal structure of a natural AvrSr50 variant was recently solved, which showed structural similarity to cupin-like proteins with a carbohydrate-binding domain. Subsequently, site-directed mutagenesis and transient expression assays were used to understand the potential mechanisms conferring the gain of virulence. *AvrSr50* mutants escaped recognition by the stem rust resistance receptor Sr50 by means of DNA insertion or stop codon loss in *AvrSr50*, or via a single amino acid substitution of the surface-exposed residue Q121 in the AvrSr50 protein (Ortiz *et al*., 2022). Furthermore, a recent study determined the key residues in the LRR domain of Sr50 responsible for AvrSr50 effector recognition and specificity by performing cell death assays in *T. aestivum* protoplasts and *Nicotiana benthamiana* leaves. These same authors engineered Sr33 from Tausch's goatgrass (*Aegilops tauschii*), which shares 80% similarity at the protein level with Sr50, to recognize AvrSr50 (Tamborski *et al*., 2022). These studies represent important steps towards understanding and monitoring rust pathogen evolution while providing avenues for *R* gene engineering in grasses.

The generation of haplo-phased reference assemblies for cereal rust isolates has enabled several recent breakthroughs in cereal rust pathogenomics and effector biology (Duan *et al*., 2022; Henningsen *et al*., 2022). A genome assembly of the highly avirulent *Pgt* isolate 21-0 led to the cloning of *AvrSr27* (Upadhyaya *et al*., 2021). In the case of *AvrSr27*, virulence for *Sr27* could be achieved experimentally or occur within the field via deletion mutations, copy number variation and expression level polymorphisms (Upadhyaya *et al*., 2021). More recently, a chromosome-scale haplo-phased reference assembly was generated for the founder isolate of the Ug99 wheat stem rust pathogen lineage (TTKSK) that enabled researchers to demonstrate that Ug99 arose due to somatic hybridization based on nuclei exchange between 21–0 and an uncharacterized *Pgt* isolate of African origin (Li *et al*., 2019). Further haplotype analysis of neighbouring *AvrSr35* and *AvrSr50* recognized effector genes revealed that both are heterozygous in the Ug99 founder lineage, highlighting the potential for single-step mutations in the pathogen to overcome their corresponding cloned, effective stem rust *R* genes (Li *et al*., 2019). This information validates the utility of Avr gene cloning studies to forecast the durability of deployed NLR genes and confirm the function of stacked race-specific *R* genes. For example, haplovariant mining analysis combined with functional characterization of natural variants of effector genes helps to understand gain-of-virulence mechanism and predict *R* gene durability and further guide *R* gene deployment strategies (Müller *et al*., 2022). Further information on the effectiveness of the different cereal rust and mildew *R* genes cloned to date is detailed in Tables S5–S9 and summarized in Figure 2 to inform potential future inclusion for pyramiding in gene stacks. The relative effectiveness of each cloned *R* gene is based on published phenotypic response data collected using diverse rust and mildew pathogen isolates from international cereal disease laboratories.

Altering the success of biotrophy utilizing naturally occurring recessive mutations

Although rare in nature, recessively inherited non-lethal loss-of-function mutations can spontaneously occur within 'susceptibility' (*S*) genes that regulate and facilitate the biotrophic feeding habit of rust and mildew pathogens (van Schie and Takken, 2014). Due to the polyploid genome of bread wheat, spontaneous recessive loss-of-function mutations in *S* genes are either tolerated or their effect is masked by functionally redundant gene homeologs (Krasileva *et al*., 2017). On average, a greater proportion of rust and mildew *R* genes are recessive in barley (26%) relative to polyploid wheat (6.7%), likely because of redundancy between homoeologous genes in wheat (Uauy *et al*., 2017). Loss-of-function mutations in *S* genes derived from polyploid species, such as bread wheat, have to date conferred pleiotropic resistance to multiple biotrophic pathogen genera and species. However, these are often accompanied by deleterious yield penalty traits due to leaf tip necrosis (Rosewarne *et al*., 2006). The best examples of loss-of-function pleiotropic resistance are two cloned partial-resistance genes, *Lr34/Yr18/Sr57/Pm38* (Krattinger *et al*., 2009) and *Lr67/Yr46/Sr55/Pm46/Ltn3* (Moore *et al*., 2015), from bread wheat that encode ABC and hexose transporters, respectively.

The most notable example in barley is *mlo*-mediated resistance to the powdery mildew fungus that has remained effective for over 50 years after its introduction into agriculture (Kusch and Panstruga, 2017). *mlo*-mediated resistance is derived from loss-of-function mutations in *Mlo* encoding a serpentine plasma membrane protein (Panstruga, 2005). The mechanism of resistance remains poorly understood, but mlo protein accumulates at powdery mildew microdomain infection sites that modulate cell actin cytoskeleton arrangement (Bhat *et al*., 2005). Several barley *mlo* alleles trigger a developmentally controlled defence mimic phenotype affecting yield; however, unlike the *Lr34/Yr18/Sr57/Pm38*, *Lr46/Yr29/Sr58/Pm39* and *Lr67/Yr46/Sr55/Pm46/Ltn3* pleiotropic partial resistance genes characterized in wheat, there is no evidence that any of the *mlo* alleles confer resistance to barley rust diseases. The adverse effects of *mlo* mutations, such as premature leaf senescence, are well documented and are consistent with Mlo having a broad role in delaying or preventing mesophyll cell death in pathogen-challenged and non-challenged leaves (Consonni *et al*., 2006; Piffanelli *et al*., 2002). However, Li *et al*. found that a 304-kb deletion-mutant (*Tamlo-R32*) at the wheat *Mlo* B-genome locus,

[Correction added on 07 August 2023, after first online publication: The term '*Lr34/Yr18/Sr57/Pm18*' has been changed to '*Lr34/Yr18/Sr57/Pm38*' in all instances in this version.]

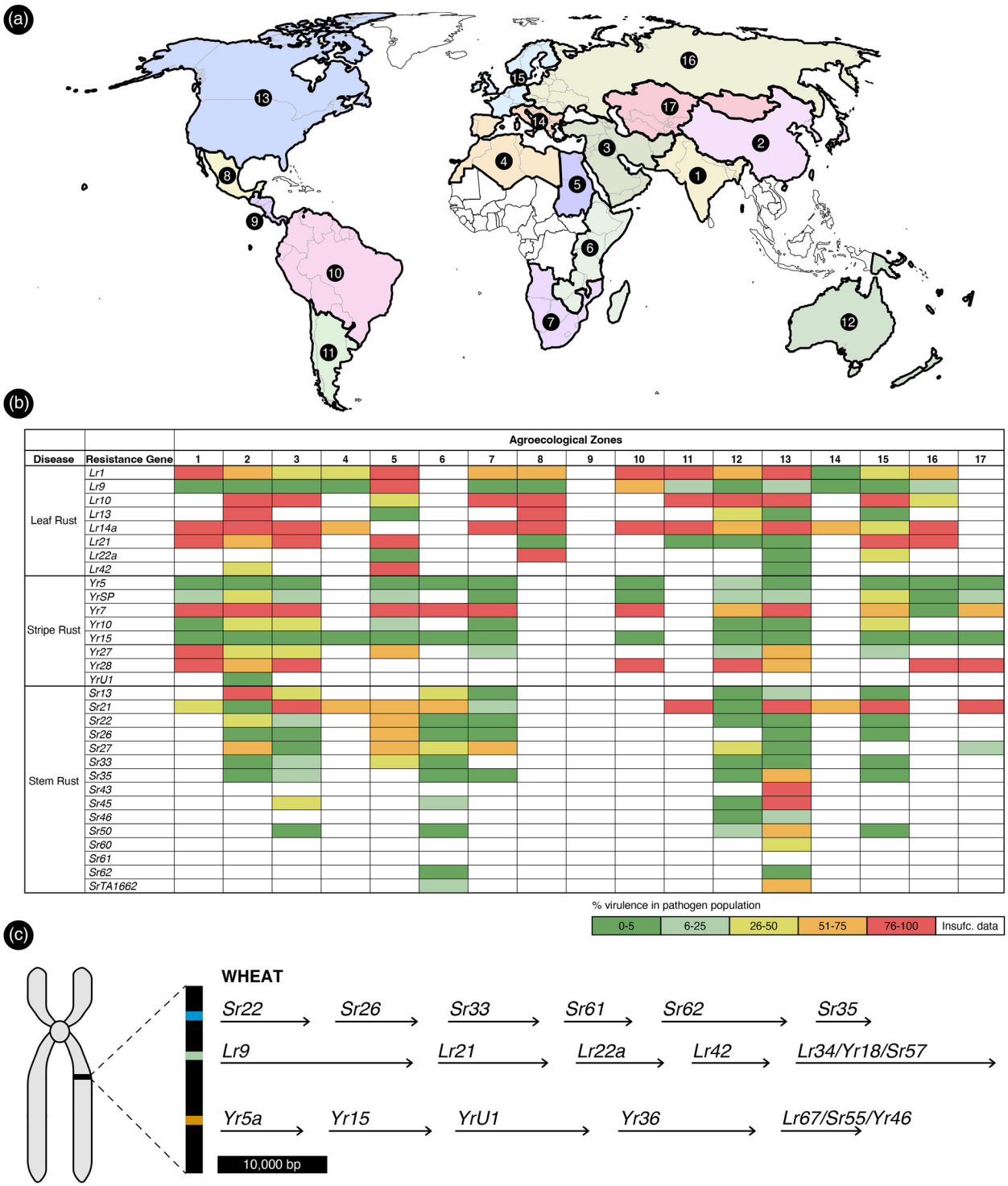

Figure 2 Efficacy of cloned resistance genes across agroecological zones. (a) Map of the world divided into 17 agroecological zones representing different target environments for wheat breeding. Adapted from Rajaram *et al*. (1994). (b) Efficacy of the 31 cloned wheat stem rust, stripe rust and leaf rust resistance (*R*) genes by agroecological zone. Colours from green to red represent virulence in the pathogen population expressed as a percentage of isolates tested against a given *R* gene that are virulent to this *R* gene. White indicates missing data (i.e. when less than five isolates from an agroecological zone have been tested against a given *R* gene). See Tables S5–S7 and S9 for further details. (c) Genes that can be combined to create polygene stacks in wheat against the three rust pathogens. The stacks were designed based on the most broad-spectrum cloned *R* genes available for each respective disease in wheat across the 17 agroecological zone(s) (Table S9). The stacks have been conceptually targeted to the same chromosome location to facilitate breeding and to ensure co-inheritance of the major dominant *R* genes and the multi-disease *R* genes *Lr34* and *Lr67*.

originally edited to create *mlo* alleles in wheat, did not result in the growth defects observed in other wheat *mlo* mutants. The absence of the *mlo*-related yield defects was not due to a deletion in the protein-coding region of *Mlo* but rather to de-repression of the neighbouring gene *Tonoplast Monosaccharide Transporter 3* (*TaTMT3B*). *TaTMT3B* is normally silenced in wild-type wheat, but the large adjacent deletion altered the local chromatin structure leading to its de-repression (Li *et al*., 2022). These desirable effects were confirmed using elite wheat cvs. under field conditions in China, and in the model species Arabidopsis. The study by Li *et al*. (2022) provides a crucial gateway to improve the utilization of *mlo*-induced resistance in cereals, other grasses, and, by extrapolation from the Arabidopsis results, in other vegetable and horticultural food crops susceptible to powdery mildew (Brown and Wulff, 2022; Spanu, 2022).

By contrast, a recent study in bread wheat suggested that a Ser/Thr kinase gene, *Puccinia striiformis-Induced Protein Kinase 1* (*TaPsIPK1*), acts as an *S* gene. The authors also cloned the *Pst* effector gene encoding PsSpg1, which targets and binds to TaPsIPK1, and showed that autophosphorylation and nuclear translocation of TaPsIPK1 is required for *Pst* virulence. The subsequent TaPsIPK1-dependent phosphorylation of the transcription factor C-REPEAT/DRE BINDING FACTOR 1d (TaCBF1d) regulated gene expression in the nucleus; however, disruption of all three *TaPsIPK1* homoeologs led to a primed host immune response conferring broad-spectrum resistance. A field assessment using geographically defined pathogen isolates and environmental conditions did not reveal any deleterious effects associated with disrupting *TaPsIPK1* via gene editing. This study highlights the ability to target multiple classes of *S* genes by gene editing to improve resistance and enhance our understanding of host–pathogen interactions (Wang *et al*., 2022a,b).

Gene stacks utilizing mechanistic diversity

The central dogma of pyramiding *R* genes states that as the number of genes introgressed, transformed or bred into a cereal variety increase, the more challenging it will be for pathogen populations to evolve corresponding virulence to multiple resistance components. Pyramiding *R* genes using transgenic and cisgenic biotechnological approaches was successful for conditioning complete late blight (*Phytophthora infestans*) resistance in potato (*Solanum tuberosum*) (Song *et al*., 2003). The *Lr34/Yr18/Sr57/Pm38* ABC transporter gene was transferred using transgenesis directly into several cereal crops including durum wheat (*T. durum*; Rinaldo *et al*., 2017), barley (Risk *et al*., 2013) and rice (Krattinger *et al*., 2016) with varying success. Severe deleterious effects, including senescence, were observed in diploid barley and rice transgenic lines expressing *Lr34*, whereas in the tetraploid durum wheat, *Lr34*-induced seedling resistance to leaf rust, stripe rust and powdery mildew (Rinaldo *et al*., 2017). This finding suggests that the deleterious effects worsened at reduced ploidy levels, which may be a feature of genes with pleiotropic functions in their endogenous polyploid background. By contrast, the cloned wheat NLR genes *Sr22*, *Sr33*, *Sr35* and *Sr45* confer race-specific resistance to *Pgt* when transformed into barley cv. Golden Promise without any noticeable agronomic effect (Hatta *et al*., 2021).

Taking advantage of technological developments in vector construction and the availability of a suite of cloned stem rust *R* genes, a stack comprising five distinct cloned *R* genes was transferred into bread wheat. The stack, referred to as the 'Big Five', comprises the cloned *Lr67/Yr46/Sr55/Pm46* partial-resistance gene and the all-stage stem rust *R* genes *Sr22*, *Sr35*, *Sr45* and *Sr50* and has been rigorously assessed for effectiveness in response to diverse global races of *Pgt*. In the T_1 progeny of three transgenic lines all five full-length genes were inherited as a single locus confirming the co-insertion of each gene. The *R* genes were transformed into the rust-susceptible bread wheat cv. Fielder and, although conferring complete immunity to seven highly virulent global *Pgt* isolates, the transgenic seedlings were susceptible to leaf and stripe rust (Luo *et al*., 2021). This study highlights the effectiveness of pyramiding four highly effective race-specific NLRs with an additional partial-resistance gene to control stem rust. The technology also demonstrates the opportunity to introduce multiple genes and/or stacks of genes into the same cv. and design either disease-specific or multi-disease *R* gene stacks containing different gene combinations for durable disease control in wheat and barley.

Based on the available rust *R* genes cloned to date in wheat, Figure 2c depicts disease-specific gene stacks that reflect a diverse mechanistic action and broad efficacy to the three rust pathogens of wheat. Both *Lr34/Yr18/Sr57/Pm38* and *Lr67/Yr46/Sr55/Pm46* were included in the leaf rust and stripe rust stacks, respectively. These genes provide incomplete yet broad-spectrum resistance to powdery mildew, redressing the reduced efficacy of cloned mildew *R* genes across the different regions in the world (Tables S8 and S9). Moreover, *Lr34/Yr18/Sr57/Pm38* and *Lr67/Yr46/Sr55/Pm46* can potentiate race-specific rust resistance genes (McCallum and Hiebert, 2022), thus likely improving the overall effectiveness of the stacks. For example, in the Swiss wheat cv. Forno, the recently cloned wheat leaf rust resistance gene *Lr14a* acts additively with the previously cloned pleiotropic APR gene *Lr34/Yr18/Sr57/Pm38*. Two additional QTLs, including the minor APR *Lr75*, constitute the leaf rust resistance complex in cv Forno, which has provided durable resistance for >30 years in Switzerland (Singla *et al*., 2017). Similarly, the race-specific *Lr13* gene is additive in the presence of *Lr34/Yr18/Sr57/Pm38* and may contribute with other QTLs to the basis of durability in the cvs. Era, Alsen and Norm (Kloppers and Pretorius, 1997; Oelke and Kolmer, 2005). In the South African cv. Kariega, durable stripe rust resistance appears to be conditioned by the APR *Lr34/Yr18/Sr57/Pm38*, the recently cloned race-specific resistance gene *Yr27* and two additional QTLs (Athiyannan *et al*., 2022; Ezzahiri and Roelfs, 1989; McDonald *et al*., 2004; McIntosh *et al*., 1992). Cloning the remaining QTLs in cvs Forno and Kariega would allow the GM stacking of their leaf and stripe rust resistance complexes to test their durability in different genetic backgrounds and environments (Athiyannan *et al*., 2022).

Interestingly, *Yr27* and *Lr13* were found to be allelic, displaying 97% sequence identity, but conferring resistance to two different types of rusts (Athiyannan *et al*., 2022). It would be highly interesting to identify the amino acids that determine pathogen specificity and see whether this would allow engineering of a dual-pathogen receptor. In a similar vein, the tandem kinases *Rwt4* and *Pm24* have been recently described to be alleles of the same gene but protecting against wheat blast and powdery mildew, respectively (Arora *et al*., 2023; Lu *et al*., 2020), whereas the leaf rust and powdery mildew *R* genes *Lr9* (Wang *et al*., 2023) and *Pm57* (Liu *et al*., 2023), encode tandem kinases fused to a von Willebrand factor A domain and share 88% amino acid homology (Liu *et al*., 2023). It can be hypothesized that *Rwt4/Pm24* and *Lr9/Pm57* are part of a three-component (indirect) guardee system, where the pseudokinase in the tandem-kinase

configuration acts as sensor of pathogen effectors. This would be similar to the ZAR1 (NLR)—RKS1 (pseudokinase)—PBL2 (kinase) case, which confers resistance to *Xanthomonas campestris* (Breit-McNally *et al*., 2022). When the pseudokinase RSK1 is 'replaced' by ZED1, another pseudokinase, there is resistance to *Pseudomonas syringae* (Wang *et al*., 2015). This is akin to the Pto and Fen kinases interacting with the NLR Prf to confer overlapping but differential ETI specificities in response to AvrPtoB (Mathieu *et al*., 2014). Therefore, the finding of the putative third component in *Rwt4*/*Pm24* and *Lr9*/*Pm57*-mediated resistance as well as their corresponding effectors may facilitate the future engineering of variants with broader-spectrum resistance or indeed dual-pathogen resistance.

Just like wheat breeders have combined distinct rust resistance genes in certain cvs. that have stood the test of time, barley breeders appear to have combined genes that provide near non-host resistance to wheat stripe rust. This immunity is genetically simple being conditioned by three QTL, including *Rps6* and *Rps7* which encode NLRs (Bettgenhaeuser *et al*., 2021; Moscou and Dawson, 2017), and *Rps8*, a physically discrete digenic complex encoding an Exo70Fx and a receptor-like kinase (Holden *et al*., 2021). Having obtained the molecular identity of these genes provides the exciting prospect of engineering this non-host immunity into wheat.

Innovative approaches to improve transformation efficiency

The recalcitrance of crop plants to Agrobacterium (*Agrobacterium tumefaciens*)-mediated transformation (ABMT) was, until recently, arguably the largest bottleneck limiting progress in plant biotechnology. This recalcitrance is largely due to the high frequency of host-Agrobacterium incompatibility explained by strong plant-induced defence responses and that Agrobacterium is predominantly adapted to infect dicot plants. The genetic basis of ABMT efficiency in plants is complex and strongly genotype-dependent. Several QTLs have been mapped in *Brassica oleracea* (Cogan *et al*., 2004) and barley (Hisano *et al*., 2017) permitting a haplotype-based assessment and selection for improved ABMT efficiency.

The options available to improve transformation efficiency are to isolate and manipulate the causal genes underlying ABMT, explore approaches to improve ABMT or devise alternative transformation systems. One approach to improve ABMT involves engineering Agrobacterium to express a type III secretion system and deliver the effectors AvrPto, AvrPtoB or HopA01 derived from the tomato (*Solanum lycopersicum*) pathogen *Pseudomonas syringae* to suppress plant defence. This technology increased ABMT efficiency by 250%–400% across the diverse plant species wheat, alfalfa (*Medicago sativa*) and switchgrass (*Panicum virgatum*; Raman *et al*., 2022). The system also permits direct delivery of a plant histone protein into somatic plant cells in a non-transgenic manner to enhance ABMT efficiency. Enhancements in ABMT efficiency have also been demonstrated in monocot species using morphogenic regulation. Expression of a construct encoding a fusion protein combining wheat GROWTH-REGULATING FACTOR 4 (GRF4) and its cofactor GRF-INTERACTING FACTOR 1 (GIF1) substantially increased ABMT efficiency and regeneration speed in monocots and dicots (Debernardi *et al*., 2020). Overexpressing *Baby boom* and *Wuschel* improved ABMT in previously recalcitrant maize inbred lines and improved transformation frequencies in sorghum (*Sorghum bicolor*) immature embryos and in rice callus (Lowe *et al*., 2016). These morphogenic enhancers could, in principle, be delivered via an Agrobacterium strain expressing a type III secretion system to further improve ABMT efficiency in cereals. An alternative approach is to deliver clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease 9 (Cas9) ribonucleoproteins (RNPs) using biodegradable, polymeric nanocomplexes via endocytosis-driven uptake into pollen grains (Gogoi *et al*., 2022). This approach was used to knock out both alleles of the cloned stem rust *R* gene *Sr50* in the homozygous wheat genotype Gladius + *Sr50*. The ability to improve ABMT efficiency or provide an alternative to tissue culture has important implications for future biotechnology applications in cereal crops recalcitrant to transformation.

Future prospects

Major advances have been made in our understanding of the molecular basis of disease resistance in crops, facilitating informed gene editing opportunities. Rapid innovations, such as gene cassettes, gene editing in *S* genes and enhanced transformation efficiency, now more than ever offer the potential to improve crop protection outcomes. Effective *R* gene combinations should, ideally, prioritize mechanistic diversity to further enhance prospects for resistance durability. Naturally occurring loss-of-function mutations within specific *S* genes involved in fungal biotrophic/parasitic habit represent promising resistance mechanisms. *S* genes often encode nutrient transporters or enzymes that are well conserved at the amino acid level, especially between closely related crop species such as wheat and barley. Opportunities for gene editing, therefore, exploit comparative genomics between closely related crop species (i.e. wheat and barley); however, this approach may be less effective when editing *S* genes in wheat derived from more distant relatives such as rice.

While rarely durable individually, it is important to continue research on NLRs and their IDs that act as pathogen effector decoys because NLRs may confer durable resistance when used in polygene stacks. Although a large repertoire of wheat and barley rust and powdery mildew *R* genes have been cloned, providing the basis for engineering powerful gene stacks against these diseases in wheat and barley, our knowledge of the efficacy of many cloned genes is still incomplete (Figure 2; Tables S5–S9). To maximize the durability of resistance, it is important to incorporate multiple genes conferring resistance against each existing pathogen isolate. In parallel, it is also important to consider the potential effect of introducing *R* genes that have a dual function and serve as susceptibility genes to promoting infection of damaging necrotrophic fungal pathogens. Therefore, a more comprehensive overview of the spectrum of resistance provided by the cloned *R* genes against contemporary pathogen isolates from around the world is required.

Generating this knowledge using existing germplasm and pathogen resources is complicated by the fact that the cloned genes are typically in different backgrounds containing other effective *R* genes. These background genes can mask the resistance conferred by the gene of interest. For example, wheat cv. Fielder, which is typically used as a transformation line to confirm the function of cloned genes, likely contains stripe rust *R* genes *Yr6* (Chen and Line, 1992) and *Yr20* (Chen *et al*., 1995), and other catalogued *Sr* and *Lr* genes yet to be determined, thus masking the resistance conferred by any cloned rust *R* gene

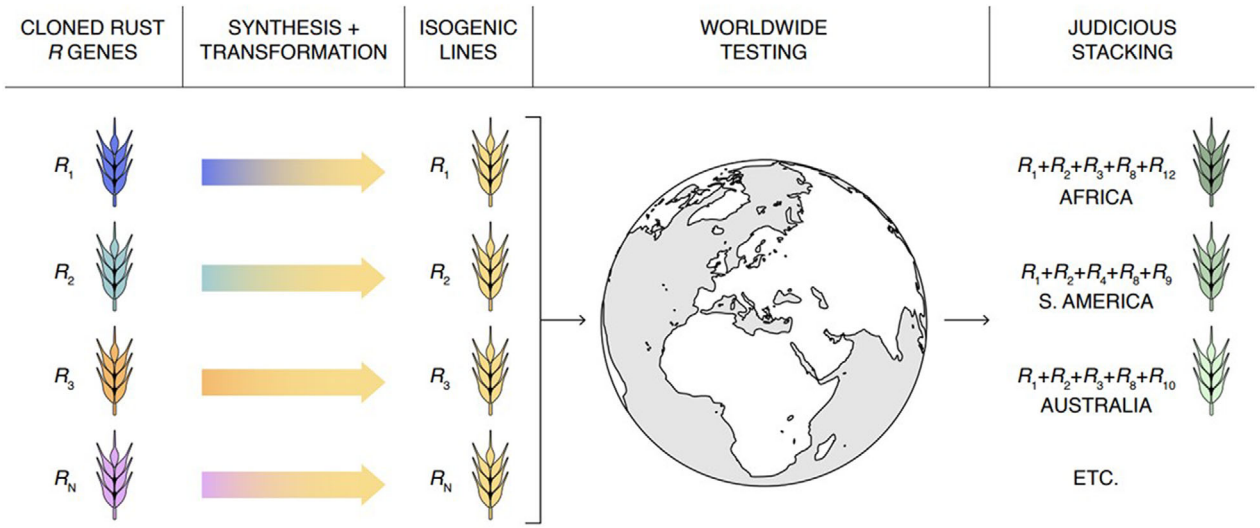

Figure 3 Creation and use of a wheat and barley isogenic library for determining the efficacy of cloned *R* genes. Cloned rust *R* genes are transformed into lines universally susceptible to either leaf rust (e.g. cv. Thatcher), stripe rust (e.g. cv. Avocet S), and stem rust (e.g. cv. Morocco). Genetically stable and publicly available homozygous isogenic lines can be shared and phenotyped around the world to determine the regional and worldwide efficacy of each gene to inform judicious stacking and deployment.

against isolates containing corresponding *Avr* genes. Today, researchers can take advantage of the low cost of DNA synthesis and the new protocols for cv.-independent transformation to incorporate 'all' cloned wheat and barley rust and mildew *R* genes into universally susceptible cvs., that is those typically used as susceptible checks. The resulting true isogenic set could then be shared with laboratories around the world for deep phenotyping. This approach would provide the information required to exercise responsible gene stewardship by permitting the judicious design of stacks that minimize the risk of selecting resistance-evading pathogen variants (Figure 3).

One important consideration for deploying transgene cassettes in wheat is the regulatory constraints and the socio-political opposition toward genetic modification technologies in wheat-growing regions around the world (Wulff and Dhugga, 2018). However, a genetically modified drought-tolerant wheat line containing the *Hb4* transcription factor gene from sunflower (*Helianthus annuus*) was deregulated in Argentina in August 2020 (González *et al.*, 2019; https://investors.biocerescrops.com/news). The flour of this wheat was later approved in Brazil, United States, Colombia, Nigeria, Australia and New Zealand, and in March 2023 the cultivation of *Hb4* wheat was approved in Brazil, thus heralding a new era for commercial biotech wheat. These developments will hopefully lower the barrier for introducing additional transgenic traits, such as for disease resistance.

Acknowledgements

We thank Xianming Chen (Washington State University), Jim Kolmer, Bob McIntosh, Simon Krattinger and Erich-Christian Oerke (University of Bonn) for helpful discussions and Emma Waller for the artwork.

Funding

This work was funded by the King Abdullah University of Science and Technology to BBHW, La Trobe University and the Alexander von Humboldt Foundation to PMD, and a China Scholarship Council fellowship to JL. JSM is recipient of the grant 'Ramon y Cajal' Fellowship RYC2021-032699-I funded by MCIN/AEI/10.13039/501100011033 and by the 'European Union NextGenerationEU/PRTR'. JSM acknowledges the support of the Junta de Castilla y León through the projects 'Escalera de Excelencia CLU-2018-04, and CL-EI-2021-04 support to the internationalisation of AGRIENVIRONMENT - Unidad Producción Agrícola y Medioambiente' of the University of Salamanca, both co-financed by the European Regional Development Fund (ERDF 'Europe drives our growth').

Conflict of interest

The authors declare they have no conflict of interest.

Author contributions

PMD and BBHW conceptualized the study and wrote the manuscript with contributions from JSM. JL performed background data consolidation for the rust efficacy tables and figures, whereas JSM collated background data for the powdery mildew efficacy table. All authors approved the final manuscript draft.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Wheat and barley grain production and trade by country.
Table S2 Wheat and barley grain end use data.
Table S3 List of cloned wheat and barley *R* genes and their mode of inheritance (adapted from Uauy *et al*., 2017, and Yu *et al*., 2022).
Table S4 List of cloned *Avr* effector genes from wheat and barley pathogens (adapted from Hafeez *et al*., 2021, and Sánchez-Martín and Keller, 2021).
Table S5 Efficacy of cloned wheat stem rust *R* genes.
Table S6 Efficacy of cloned wheat stripe rust *R* genes.
Table S7 Efficacy of cloned wheat leaf rust *R* genes.
Table S8 Efficacy of cloned wheat powdery mildew *R* genes.
Table S9 Efficacy summary for cloned wheat rust and powdery mildew *R* genes.

