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Lr21 diversity unveils footprints of wheat evolution and its new role in broad-spectrum leaf rust resistance

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Abstract

Aegilops tauschii, the progenitor of the wheat D genome, contains extensive diversity for biotic and abiotic resistance. *Lr21* is a leaf rust resistance gene, which did not enter the initial gene flow from *Ae. tauschii* into hexaploid wheat due to restrictive hybridization events. Here, we used population genetics and high-resolution comparative genomics to study evolutionary and functional divergence of *Lr21* in diploid and hexaploid wheats. Population genetics identified the original *Lr21*, *lr21-1* and *lr21-2* alleles and their evolutionary history among *Ae. tauschii* accessions. Comparative genetics of *Lr21* variants between *Ae. tauschii* and cultivated genotypes suggested at least two independent polyploidization events in bread wheat evolution. Further, a recent re-birth of a unique *Lr21-tbk* allele and its neofunctionalization was discovered in the hexaploid wheat cv. Tobak. Altogether, four independent alleles were investigated and validated for leaf rust resistance in diploid, synthetic hexaploid and cultivated wheat backgrounds. Besides seedling resistance, we uncover a new role of the *Lr21* gene in conferring an adult plant field resistance. Seedling and adult plant resistance turned out to be correlated with developmentally dependent variation in *Lr21* expression. Our results contribute to understand *Lr21* evolution and its role in establishing a broad-spectrum leaf rust resistance in wheat.

KEYWORDS
Aegilops tauschii, exotic diversity, resistance genetics, R-gene, *Triticum aestivum*

1 | INTRODUCTION

Plants and pathogens are in a continuous tug-of-war for their survival. The leaf rust fungus *Puccinia triticina* is one of the most persistent pathogens of wheat globally (Savary et al., 2019). It is an obligate parasite that can produce infectious urediniospores as long as infected leaf tissue remains alive, which may result in a higher mutation rate and reduction in wheat production annually (Bolton, Kolmer, & Garvin, 2008; Kolmer, 2005). Wheat has evolved resistance (*R*) genes which act as whistleblowers that trigger host immunity at the onset of pathogen attack (Dilbirligi, Erayman, Sandhu, Sidhu, & Gill, 2004; Ellis, Lawrence, Luck, & Dodds, 1999). Most *R* genes impart qualitative

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resistance, which prevents the pathogens from completing their life cycle in the host. In crop monocultures, such resistances are not durable but often rapidly overcome through selection for gain-of-virulence in the pathogen population (Boyd, Ridout, O'Sullivan, Leach, & Leung, 2013). Intriguingly, a quantitative or partial resistance has been found to be more stable as the co-existence of host and pathogen is possible over long periods (Giannakopoulou et al., 2015). Therefore, the identification of leaf rust resistance genes and their functional evaluation are of prime importance for breeding resilient wheat varieties.

A genetic understanding of host-pathogen interactions is essential for developing and maintaining durable disease resistance. In the past, Flor's work laid the foundation to explain host-pathogen interactions based on the gene-for-gene hypothesis (Flor, 1942). Later on, R protein interactions with pathogen-related products known as avirulence (avr) effector proteins in modulating resistance reactions were identified (Shan, Cao, Dan, & Tyler, 2004; Van Kan, Van Den Ackerveken, & De Wit, 1991). An absence of the *R* gene or *avr* gene was linked with the non-recognition of pathogen and host susceptibility. Jones and Dangl illustrated the host-pathogen interaction further by a zig-zag model. According to this, a pathogen infects a host and delivers *avr* effector proteins which target and disable the host immune system to facilitate pathogen growth (Jones & Dangl, 2006). To protect themselves, plants have evolved *R* genes the products of which recognize these effector molecules through their interaction with immune system components. A typical defence response involves a host hypersensitive and sudden cell death at the point of infection by the accumulation of H_2O_2 or related substances (Dangl & Jones, 2001; Pellinen, Korhonen, Tauriainen, Palva, & Kangasjärvi, 2002). These reactions are usually speedy and strong and do not permit biotrophic pathogens like leaf rust to grow and multiply on their hosts. As leaf rust populations can evolve faster than their host due to their short life cycle and high spore production, new virulences may arise quickly resulting in the existing resistance genes being overcome.

Not all but the majority of *R* genes encode nucleotide-binding site (NBS) and leucine-rich repeat (LRR) proteins, also known as NLRs (Kourelis & Van Der Hoorn, 2018). It has been shown that the LRR domain of NLRs is involved in the recognition of pathogen effectors, while the NBS domain mediates protein oligomerization for initiating a signal transduction that triggers host immunity (Cesari, 2018; DeYoung & Innes, 2006; van Wersch, Tian, Hoy, & Li, 2020). Recent advances have suggested more complex mechanisms of host-pathogen interactions at the levels of transcription, post-transcription, post-translation and molecular interaction. In addition, additional players like helper NLRs have been identified which provide downstream signalling function in activating host immunity (Bonardi et al., 2011; Jubic, Saile, Furzer, El Kasmi, & Dangl, 2019). These factors altogether support plants to evolve new variants of defence mechanisms conferring quantitative (partial) resistance against a pathogen or multiple pathogens simultaneously (van Wersch et al., 2020). Gene annotation based on whole genome sequence data predicted around 150 and 1,500 NLR genes in diploid *Arabidopsis thaliana* and hexaploid wheat, respectively (Meyers, Kozik, Griego, Kuang, & Michelmore, 2003; Steuernagel et al., 2020). A species-wide analysis identified pronounced variation in the number, arrangement and domain combinations between genotypes suggesting a rapid birth and death of *R* genes (Collier, Hamel, & Moffett, 2011; Michelmore & Meyers, 1998). For leaf rust resistance in wheat, 80 (*Lr1–Lr80*) mostly qualitative *R* genes have been identified to date (Kumar et al., 2021). A few of these genes, namely *Lr34*, *Lr46*, *Lr67*, *Lr68* and *Lr75* confer partial resistance against leaf rust (Singla et al., 2017). Among these, *Lr34* and *Lr67* have been functionally characterized and found to express an ATP-binding cassette (ABC) and a hexose transporter protein, respectively (Krattinger et al., 2009; Moore et al., 2015). On its own, *Lr34* has supported a broad-spectrum durable resistance against multiple pathogens in wheat for more than 50 years (German & Kolmer, 1992; Risk et al., 2012).

Lr21 was one of the first leaf rust resistance genes identified from an Iranian *Aegilops tauschii* accession and transferred to bread wheat in the 1970s (Rowland & Kerber, 1974). Previously, the *Lr21* gene was cloned and found that to encode a typical NLR protein (Huang et al., 2003). Later, the authors created a chimeric *Lr21* resistance allele in wheat by crossing two non-functional haplotypes, H1 (*lr21*-Fielder, 1 bp deletion in exon 2) and H2 (*lr21*-Wichita, 2 bp deletion in exon 1) screening for resistant progeny (Huang et al., 2009). They were, however, unable to trace these haplotypes in the *Ae. tauschii* gene-pool. Therefore, the origin, evolutionary and functional divergence of *Lr21* alleles from diploid progenitor species to hexaploid wheat have until now remained elusive. Further, the function of *Lr21* had until now primarily been studied in seedling resistance, while its broader agricultural relevance in restricting adult plant field resistance had not yet been fully explored.

In the present study, we analysed the evolutionary divergence of *Lr21* alleles in diploid *Ae. tauschii*, synthetic hexaploid accessions and cultivated wheat gene-pool. We phenotypically characterized *Lr21* alleles in diploid and hexaploid backgrounds, and compared *Lr21* resistance in mediating seedling and adult plant field resistance. Here, we present a unique diversity and evolutionary study of *Lr21* alleles as well as their function in conferring leaf rust resistance under controlled and field conditions. Our results will help to devise new strategies for establishing durable leaf rust resistance in wheat.

2 | RESULTS

2.1 | Allelic diversity and re-birth of a unique *Lr21* allele in wheat cultivar Tobak

The genetic analysis of leaf rust resistance in an advanced backcross (AB) population designated as B22 revealed a major QTL for resistance on the distal arm of chromosome 1DS (Naz, Kunert, Lind, Pillen, & Léon, 2008). We observed a characteristic hypersensitive resistance reaction in the synthetic hexaploid donor parent Syn022L compared to a high level of susceptibility in the recurrent parent Batis against the leaf rust isolate 77WxR. These resistance and susceptible

infection reactions segregated among the AB-lines (Figure S1). In a previous study, *Lr21* was identified as the resistance gene at this locus using a positional cloning approach (Huang et al., 2003). To validate if the identified resistance in Syn022L was controlled by *Lr21* or not, we combined genome-wide mapping and candidate gene analysis. We genotyped the B22 population using the 15 K Single Nucleotide Polymorphism (SNP) array and compared the genotypic and phenotypic data using an AB-QTL analysis strategy (Figure S2). Marker-by-phenotype analysis revealed six associated SNPs in the targeted region on the distal arm of chromosome 1DS. The relative ordering of these SNPs was determined on the physical genomic map of wheat. The most significant SNP marker RAC875_c10925_1887 showed co-segregation of the Syn022L and Batis alleles with the resistant and susceptible infection types, respectively. This marker was placed 271 kb from the *Lr21* gene. Next, we amplified and sequenced the full-length genomic fragment of the *Lr21* gene in Syn022L and cultivar Batis. Sequence comparison identified a critical mutation in exon 2 where the nucleotide adenine was deleted in cultivar Batis at position +1,773 bp. This deletion resulted in a frame-shift to stop codon in cultivar Batis whereas Syn022L harbours a functional *Lr21* resistance allele (Figure S3). These data suggest that the seedling resistance among AB-lines underlie an *Lr21* allele derived from the synthetic wheat accession Syn022L.

Next, we explored the population-wide *Lr21* allelic variation present in the diploid *Ae. tauschii* and in hexaploid wheat genotypes. For this, we compared the *Lr21* gene sequence in 140 *Ae. tauschii* accessions, four selected wheat cultivars and two synthetic wheat accessions (Syn022L and Syn086L). The synthetic hexaploid wheat Syn022L and the *Ae. tauschii* genotypes BW_20983 and BW_214616 were found to harbour the full-length functional *Lr21* resistance allele (Figure S4). These data suggest that the functional *Lr21* allele in Syn022L originated from the D genome progenitor *Ae. tauschii* prior to its hybridization with tetraploid emmer wheat (*Triticum turgidum*) to form hexaploid bread wheat. In addition, we identified two loss-of-function *lr21* alleles designated as *lr21-1* and *lr21-2* among the *Ae. tauschii* accessions, synthetic wheat Syn086L and the cultivars Zentos, Chinese Spring and Batis, respectively (Figure 1a). According to this, *lr21-1* carries the di-nucleotide deletion "CA" at position +761 bp in exon 1, which results in a pre-mature stop codon (Figure S5). The second allele, *lr21-2*, carries an adenine deletion at position +1,773 bp as described above in cultivar Batis (Figure S6). Further, we performed *Lr21* allele mining in a diverse collection of wheat cultivars using gene-specific functional haplotype sequencing. Most of the genotypes carried loss-of-function alleles, *lr21-1* (24.7%) and *lr21-2* (74.3%) except cultivar Tobak (Figure S7). Full-length *Lr21* sequencing of Tobak revealed a previously unkown *Lr21-tbk* allele in wheat. Unlike all others, the *Lr21-tbk* allele appears to be due to several spontaneous mutations in exon 2 and 3, which resulted in the recovery of the *Lr21* open reading frame and a putative re-birth of a functional *Lr21* allele (Figure 1b). The original *Lr21* resistance encoding allele from Syn022L and *Ae. tauschii* encodes a protein of 1,080 amino acids containing an NB-ARC domain (Figure 1c). The loss-of-function alleles *lr21-1* and *lr21-2* revealed truncated proteins of 150 and 380 amino acid residues, where the essential NB-ARC domain was completely or partly lost (Figure 1d). *Lr21-tbk* encodes a full-length protein (1,080 amino acids) revealing three amino acid substitution mutations of which two are located in the NBS domain (Figure 1e, Figure S8). In summary three allelic variants of *Lr21* among the *Ae. tauschii* accessions were discovered and traced in hexaploid wheat. Furthermore, a unique *Lr21-tbk* allele in the wheat cultivar Tobak was discovered.

2.2 | Population genetics reveals evolutionary divergence of *Lr21* alleles

Next, we combined population genetics of *Ae. tauschii* accessions and the geographic distribution of *Lr21* alleles to gain insight into the evolutionary divergence of *Lr21* alleles. Initially, ancestry coefficient values of individual genotypes were calculated using 317 genome-wide SNP markers in 151 *Ae. tauschii* accessions (Arora, Cheema, Poland, Uauy, & Chhuneja, 2019). Next, the population structure was interpolated with genotype location (origin) and allelic background to assort the genotypes on a geographic map (Figure S9). The loss-of-function allele *lr21-1* revealed a broader distribution whereas the functional *Lr21* allele was located south of the Caspian Sea in Iran. In addition, two genotypes carrying the same functional *Lr21* allele were found in Azerbaijan and Turkey. Notably, a single accession (BW_01028) was identified, originating from Georgia that carries the *lr21-2* loss-of-function allele (Figure 2a). Principal component analysis was performed to elucidate the genetic relatedness of individual *Ae. tauschii* accessions. These results showed that the population was divided into three sub-groups, but all genotypes carrying the *Lr21* allele clustered in one major group. Strikingly, the *Ae. tauschii* accession (BW_01028) carrying the *lr21-2* allele was found genetically distant to all groups (Figure 2b). In population-based phylogenetic analysis, this genotype clustered in a major clade giving hint to a common ancestry of the *Lr21* and *lr21-2* alleles (Figure 2c). The precision of differentiation between the genotypes was high, as the average au probability was 0.977, where the 95% quantile ranged from 0.86 to 1 (100 bootstraps were performed, Figure S10). Thereafter, we performed a phylogenetic analysis based on *Lr21* sequence variations within the *Ae. tauschii* population as well as in selected hexaploid wheat cultivars harbouring different *Lr21* alleles. All genotypes carrying the *Lr21* allele clustered together suggesting a common ancestor of the *Lr21* and *lr21-2* alleles. Interestingly, the newly evolved *Lr21-tbk* was found closest to the *lr21-2* allele. Chinese Spring clustered with a separate clade of genotypes harbouring the *lr21-1* allele (Figure 2d). Based on allele frequency, *lr21-1* was found to be the most frequent whereas *lr21-2* appeared to be a rare allele. The gain of function *Lr21* allele was identified in around 10% *Ae. tauschii* accessions (Figure 2e). These data suggest at least two independent mutation events in the divergence of *Lr21* alleles prior to their interspecific hybridization. Moreover, *Ae. tauschii* accessions carrying the loss-of-function alleles *lr21-1* and *lr21-2* appeared to be involved in the establishment of hexaploid wheat.

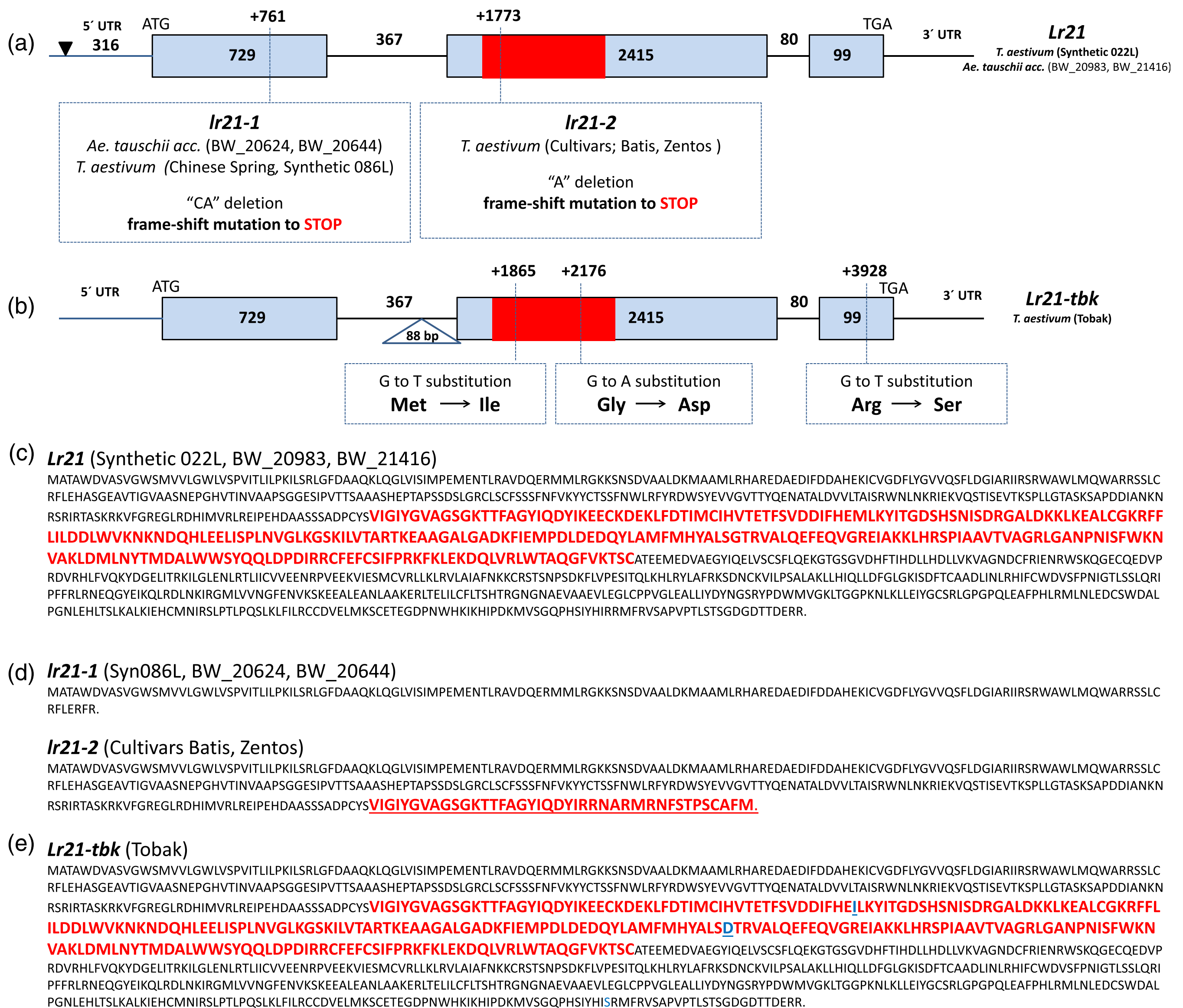

FIGURE 1 *Lr21* allelic variation in *Ae. tauschii* accessions, synthetic and cultivated hexaploid wheat genotypes. (a) Structure of the *Lr21* gain-of-function allele from synthetic hexaploid wheat accession Syn022L and related *Ae. tauschii* accessions. Causual mutations of the loss-of-function *lr21-1* and *lr21-2* alleles are indicated which are relative to transcription initiation site (black triangle) according to Huang et al. (2003). (b) Structure and sequence variation of the newly established *Lr21-tbk* allele in cultivar Tobak. (c) Predicted protein sequence of Lr21. Red color marks the NBS domain. (d) Predicted truncated protein sequence of *lr21-1* and *lr21-2*. (e) Predicted protein sequence of the Tobak *Lr21-tbk* allele. Amino acid substitutions are underlined and marked in blue colour. Met: Methionine, Ile: Isoleucine, Gly: Glycine, Arg: Arginine, Ser: Serine

2.3 | Functional characterization and new-specificities of *Lr21* resistant alleles

Diploid and hexaploid wheat genotypes were selected based on *Lr21* allelic variations for functional characterization along with a susceptible control. Detached leaves were inoculated with the multi-virulent isolate 77WxR of *P. triticina* possessing the *Avr-Lr21* factor. This detached leaf assay resulted in the formation of sporulating uredinia on *T. aestivum* cvs. Monopol and Batis (Figure 3a). As expected, Syn022L carrying the *Lr21* allele showed no sporulation, but developed resistance reactions associated with the formation of small chlorotic spots (Figure 3b). Interestingly, the cultivar Tobak harbouring the newly detected *Lr21-tbk* allele was largely unaffected and showed some faint chlorosis (Figures S11 and S12). The frequency of uredinia formation by *P. triticina* 77WxR on leaves of *Ae. tauschii lr21-1* (genotypes BW_20624 and BW_20644) was lower, nevertheless, the pathogen produced a high number of spores; uredinia were smaller and were surrounded by a small chlorotic halo on BW_20624. Leaves of genotypes BW_20983 and BW_21416 carrying the original resistance encoding *Lr21* allele were completely free of symptoms. Successful infections of *P. triticina* 77WxR prolonged the vitality of the detached leaves (Figure S13). The results on detached leaf assays confirmed the infection

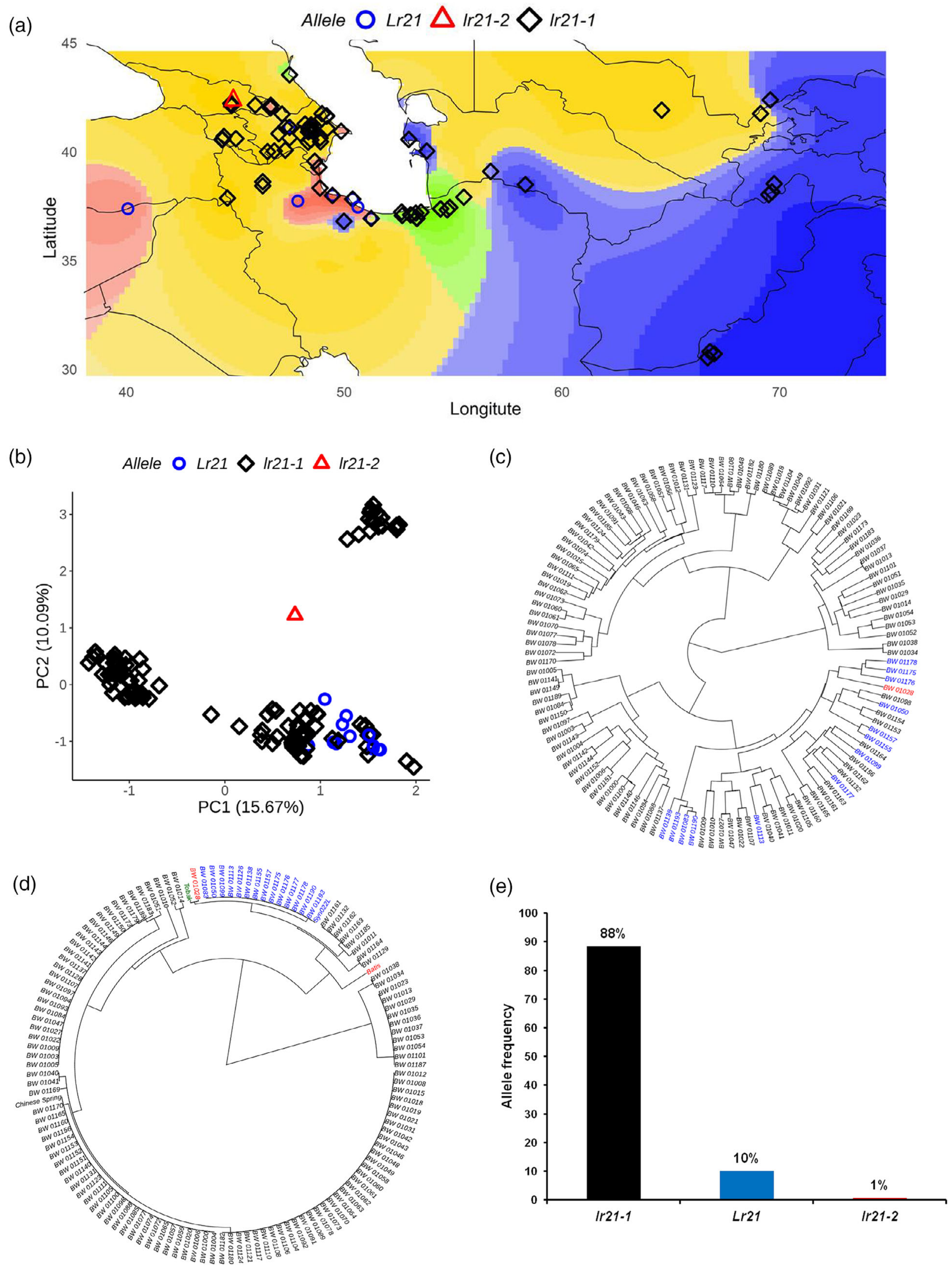

FIGURE 2 Population genetics and evolutionary divergence of *Lr21*. (a) Geographic distribution of the *Ae. tauschii* population relative to *Lr21* alleles. (b) PCA of *Ae. tauschii* accessions based on genome-wide data. Different *Lr21* alleles are indicated by differently colored symbols. (c) Phylogenetic relationship of *Ae. tauschii* accessions based on genome-wide SNP data, black, blue and red genotypes carry *lr21-1*, *Lr21* and *lr-21-2* alleles, respectively. (d) Phylogenetic relationship based on *Lr21* sequence variation among *Ae. tauschii* accessions and selected hexaploid wheat genotypes. (e) *Lr21* allele frequency in *Ae. tauschii* population [Colour figure can be viewed at wileyonlinelibrary.com]

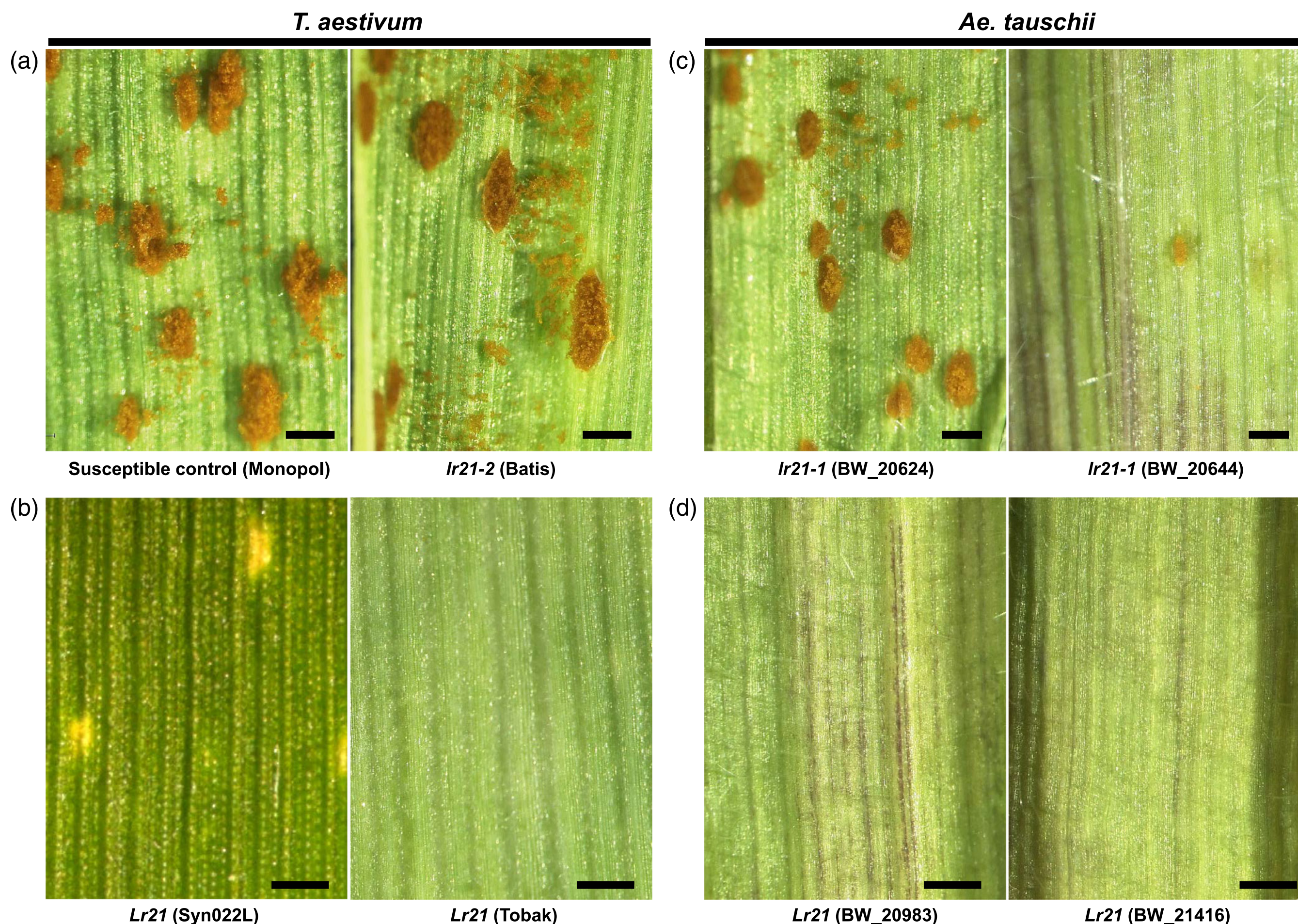

FIGURE 3 Seedling resistance of *Lr21* alleles against the leaf rust pathogen isolate 77WxR. (a and b) Susceptible infection types in hexaploid wheat cultivars Monopol (control) and Batis carrying the *lr21-2* allele (a) and two independent *Ae. tauschii* accessions (BW_20624, BW_20644) harboring *lr21-1* (b). (c and d) Resistance reaction of hexaploid wheat genotypes Syn022L and Tobak carrying the original *Lr21* and *Lr-tbk* alleles, respectively (c), and reaction of two independent *Ae. tauschii* accessions (BW_20983, BW_21416) carrying the original *Lr21* allele (d). Scale bars represent 1 mm [Colour figure can be viewed at wileyonlinelibrary.com]

types on seedlings and also the differences in uredinia size (Table S1). These data suggest that diploid and hexaploid wheat genotypes showed *Lr21* allele specific responses in term of susceptible and resistance infection types against leaf rust isolate 77WxR that carries the *AvrLr21* factor.

In addition, we inoculated cv. Batis, Syn086L and Syn022L with the isolate 77WxR to decipher the resistance and susceptible reactions of the *Lr21*, *lr21-1* and *lr21-2* alleles at the cellular level. Mesophyll cells were evaluated 72 and 168 hr after inoculation (hai) to monitor the development and spread of fungal structures within the leaf tissue; hyphae, haustorial mother cells and uredospores were observed. On cultivar Batis, carrying the loss-of-function *lr21-2* allele, the superficial uredospores had established intercellular hyphae (with haustoria, not visible) 72 hai which increased in number by 168 hai (Figure 4a). These features correspond to the high susceptibility in Batis to isolate 77WxR. Similar results were observed in the synthetic wheat Syn086L harbouring the loss-of-function *lr21-1* allele (Figure 4b). In contrast, the fungal structures were restricted to primary vesicles in stomatal cavities and were unable to spread in the synthetic wheat Syn022L reaction (Figure 4c). Auto-fluorescence, a marker of host resistance response to pathogen was detected at 72 hai within the mesophyll cells around the infection sites in Syn022L, which was absent in susceptible genotypes Batis and Syn086L. The quantification of haustorial mother cells at 72 hai and uredospore pustules generated per mm^2 were dramatically lower in Syn022L as compared to Batis and Syn086L (Figure S14). Taken together, these data suggest that *Lr21* mediates a hypersensitive resistance reaction at the seedling stage against isolate 77WxR.

2.4 | *Lr21* confers complete resistance at the seedling and partial resistance at the adult plant stage under field conditions

Our initial mapping revealed a common QTL for seedling and adult plant field resistance (Kolmer et al., 2008). This led us to hypothesize additional functions of *Lr21* beyond seedling resistance. In the present study, we compared the seedling and adult plant field resistance

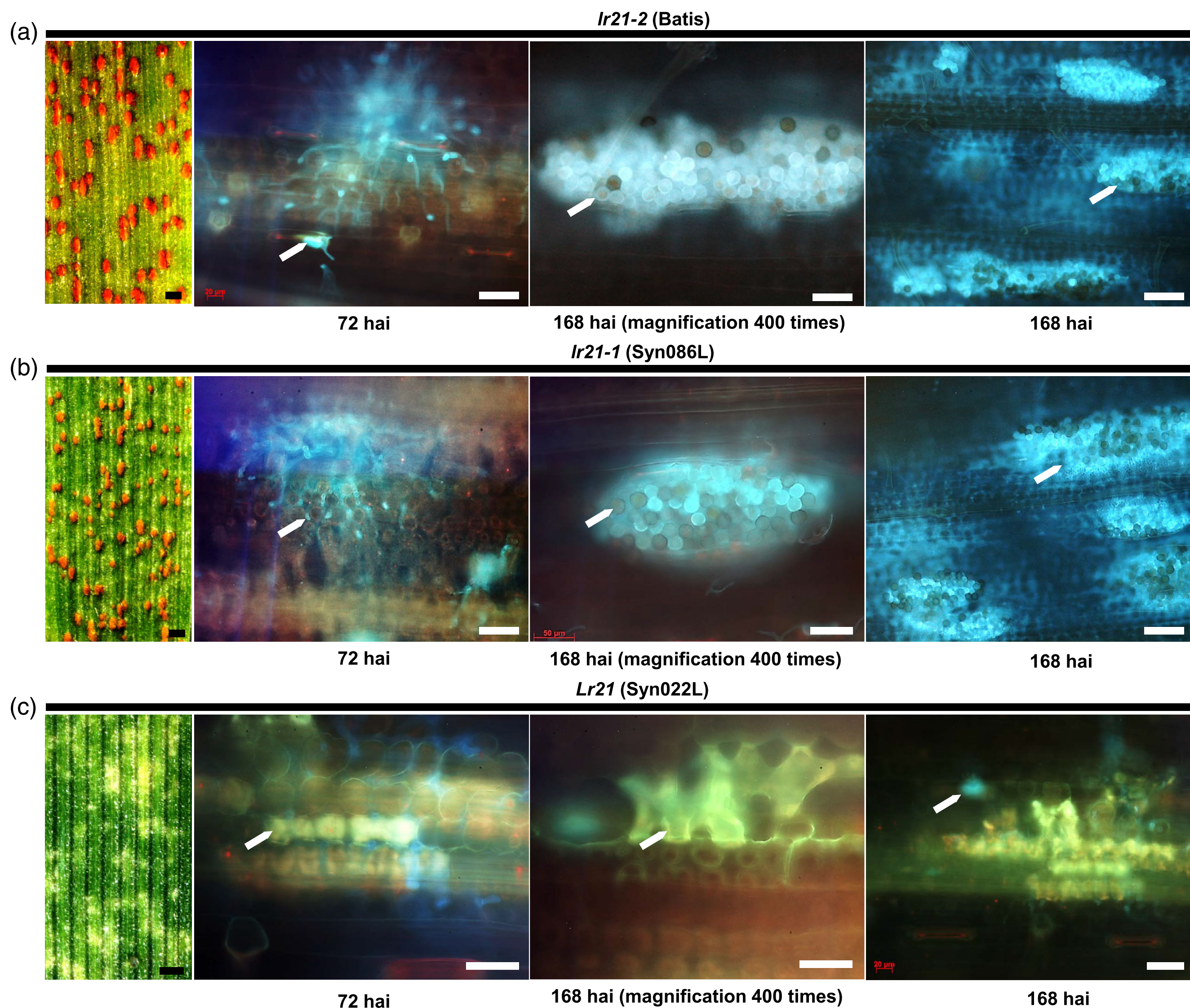

FIGURE 4 Host-pathogen interaction and functional validation of *Lr21*. Leaf symptoms and progression of leaf rust disease and resistance reaction at 72 hai and 168 hai in Batis carrying *lr21-2* (a), Syn086L carrying *lr21-1* (b) and Syn022L carrying *Lr21* allele (c). hai: hour after inoculation. Arrows in pictures taken at 72 hai show the appressorium (a), haustorial mother cell (b) and mesophyll cell showing autofluorescence (c). Arrows at 168 hai (magnification 400 times) show uredospores (a and b) and autofluorescence around the infection site (c). Arrows at 168 hai show uredospore pustules (a and b) and the appressorium (c). Bars of leaf segment pictures represent 1 mm, bars in pictures taken 168 hai (magnification 400 times) represent 50 μm and at 168 hai 200 μm (a and b) and 50 μm (c). Contrast and brightness have been adjusted [Colour figure can be viewed at wileyonlinelibrary.com]

dataset of the population B22 to elucidate the role of *Lr21* in mediating broad-spectrum resistances against leaf rust. A comparative QTL analysis was made using seedling resistance and adult plant field resistance data from 10 environments and high-resolution SNP data. This analysis revealed a single major peak on chromosome 1DS at the position of the *Lr21* gene for seedling as well as for adult plant field resistance. Notably, the association peak and the most significant SNP marker RAC875_c10925_1887 (SNP-Lr21) were common for both resistance types (Figure 5a,b). Marker-trait association analysis using a gene-specific *Lr21* marker validated the superiority and consistent effect of the *Lr21* allele for leaf rust resistance across different environments (Figure 5c,d). The *Lr21* resistance allele explained a larger proportion of the genetic variance for seedling resistance (59.9%) as compared to adult plant resistance (39.9%) indicating a partial resistance under field conditions. Next, we monitored candidate AB-lines carrying *Lr21* and *lr21-2* alleles for their characteristic differences in the reaction to leaf rust under field conditions. Intriguingly, small amounts of sporulation and chlorotic resistance spots were observed in AB-lines (WW22-75, WW30-63) carrying the *Lr21* resistance allele from Syn022L (Figure S13A,B). These rust symptoms are characteristic of a partial leaf rust resistance. In contrast, evident susceptibility and rust sporulation were observed in AB-lines WW31-19 and WW28-02 carrying the susceptible *lr21-2* allele from cultivar Batis (-Figure S15C,D). These data indicate a more divergent role of *Lr21* in

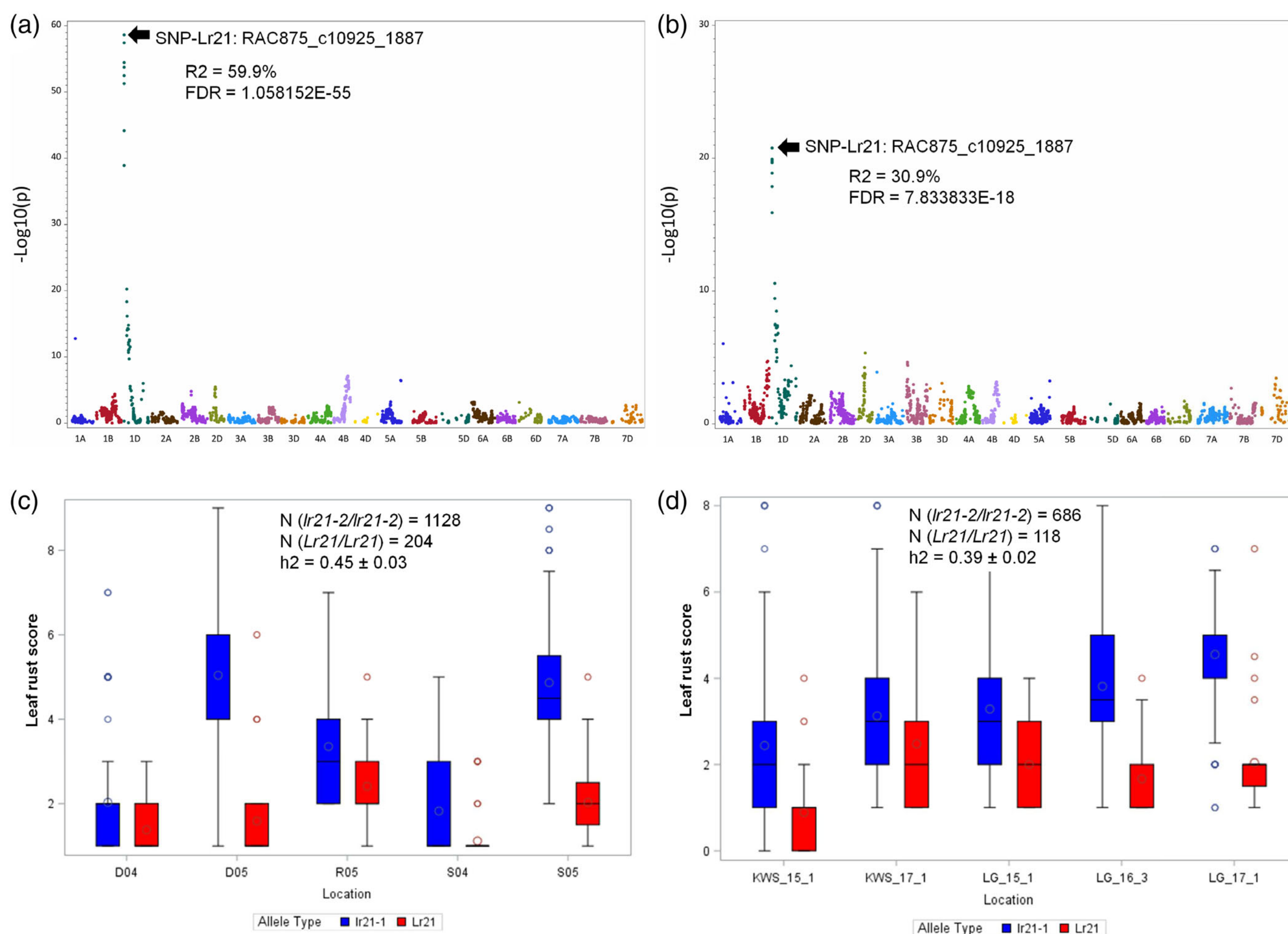

FIGURE 5 Comparative genetic mapping of seedling and adult plant leaf rust resistances in the advanced backcross population B22. (a and b) Marker trait associations (Manhattan plots) for seedling (a) and adult plant field resistance (b). C-D) Box-plots showing effects of *Lr21* and *lr21-2* alleles in population B22 under field evaluation I (years 2004, 2005) (c), and field evaluation II (years 2015, 2016, 2017) for the validation of leaf rust adult plant field resistance (d) [Colour figure can be viewed at wileyonlinelibrary.com]

mediating a developmentally dependent resistance against leaf rust. To further analyse this, we measured mRNA expression of *Lr21* in the non-infected leaves of resistant AB-line WW22-75 and the susceptible recurrent parent Batis across different developmental timepoints using qRT-PCR analysis. *Lr21* mRNA was highly abundant in the AB-line WW22-75 at the seedling stage (2 weeks), but decreased significantly at 4 weeks, and a further dramatic decrease was observed in 8 and 12 week old plants. A similar pattern, but with substantially lower amounts of transcript, was observed in cultivar Batis across 2, 4, 8 and 12 weeks (Figure 6). These data showed a stage-specific *Lr21* mRNA expression variation, where a higher expression at the seedling stage appears to correlate with a hypersensitive resistance reaction while a lower *Lr21* expression later in development correlates with partial leaf rust resistance.

3 | DISCUSSION

The creation of hexaploid wheat is one of nature's miracles where three independent grass species hybridized in two consecutive steps. Such allo-polyploidization events often present genetic bottlenecks resulting in the erosion of many alleles existing in the diploid species. *Lr21* is a typical case of such genetic erosion as a limited member of progenitor genotypes appeared to be involved in the polyploidization of bread wheat. An Iranian *Ae. tauschii* accession carrying the *Lr21* resistance allele was identified and transferred to bread wheat in the 1970s (Rowland & Kerber, 1974). The pioneering work of the group of Prof. B. Gill on gene cloning in wheat showed that *Lr21* encodes a typical NLR protein (Huang et al., 2003). In a follow-up study, they analysed allelic variation and compared the sequence of *Lr21* and *lr21* alleles in 24 wheat cultivars and 25 *Ae. tauschii* accessions to trace-back the origin of two non-functional variants in *lr21-f* (Fielder) H1 (1 bp deletion in exon 2) and *lr21-w* (Wichita) H2 (2 bp deletion in exon 1). The *lr21-w* was identified in *Ae. tauschii* but they were unable to trace the *lr21-f* allele within the 25 *Ae. tauschii* accessions (Huang et al., 2009). Due to this, the primary questions behind its allelic and evolutionary divergence remained unanswered. In the present study, we performed population-wide sequence-based analysis of *Lr21* allelic variants and evaluated their evolutionary divergence across *Ae. tauschii* accessions relative to hexaploid wheat cultivars. Along with

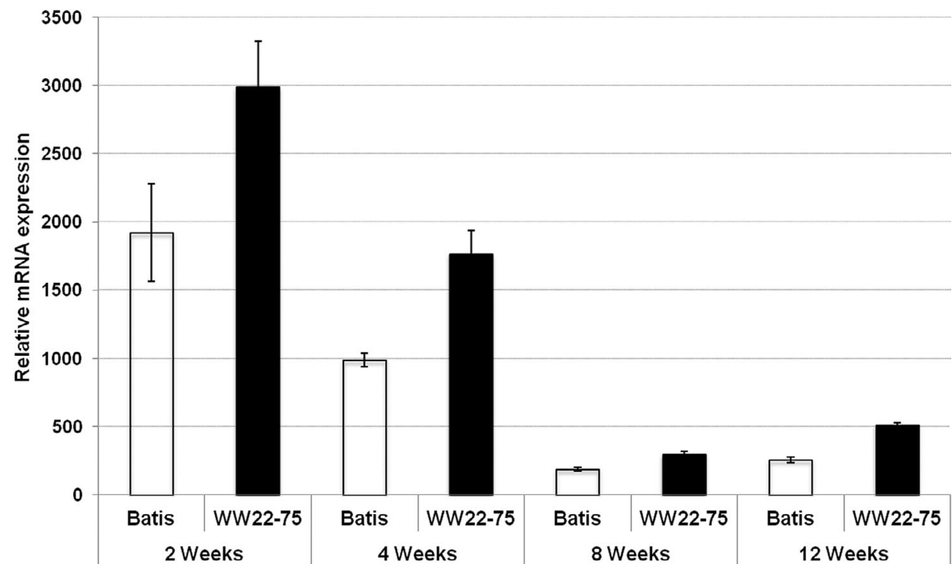

FIGURE 6 Quantitative RT-PCR analysis in AB-line WW22-75 carrying the *Lr21* allele from Syn022L relative to the recurrent parent Batis as a control. Bars represent standard error relative to the mean of the biological replicates ($n = 2$) and technical replicates ($n = 3$) in each genotype

the *Lr21* allele, we successfully identified both of the original causal variants of *lr21-w* and *lr21-f* among the *Ae. tauschii* accessions and designated them *lr21-1* and *lr21-2*, respectively. Although *lr21-1* and *lr21-2* showed that same causal mutation as reported previously in *lr21-w* and *lr21-f* alleles, we designated these differently because of their origin from *Ae. tauschii* accessions and their genetic background. The *lr21-2* allele was identified in a previously unknown *Ae. tauschii* accession from Georgia. In addition, we uncovered a recent evolutionary event and the re-birth of a new functional *Lr21-tbk* allele in the wheat cultivar Tobak. The origin of *Lr21-tbk* is still an unsolved mystery due to the accumulation of several mutations and the recovery of the *Lr21* function independent from the existing alleles originating from *Ae. tauschii*. The previous hypothesis of *Lr21* recovery proposed by Huang et al. (2009) in *Ae. tauschii* due to outcrossing also cannot explain the evolution of the *Lr21-tbk* allele due to its novelty. The cultivar Tobak has been considered one of the most successful European winter wheat cultivars due to its adult plant field resistance to leaf rust and high yielding characteristics.

Our data on population genetics, allele frequency and phylogenetic analyses predicted several independent mutation events resulting in *Lr21* allelic diversity. The non-functional *lr21-1* appeared to be the primary allele in *Ae. tauschii* accessions, which may have evolved to a gain-of-function *Lr21* allele due to a spontaneous mutation at H1 in exon 1. In the *Lr21* functional allele a second spontaneous mutation in exon 2 may have resulted in the development of a rare *lr21-2* allele identified in one *Ae. tauschii* accession originating from Georgia. Notably, this rare *lr21-2* allele was identified in the winter wheat cultivar Batis, which is a European winter wheat cultivar. In contrast, the *lr21-1* allele was identified in the spring wheat cultivar Chinese Spring. This distribution of original *Ae. tauschii lr21* alleles in cultivated genotypes suggest at least two independent polyploidization events in the establishment of hexaploid wheat. In line with these observations, previously two distinct alleles of the *A1* locus were identified in *Ae. tauschii* accessions, both of which were present in hexaploid wheat (Talbert, Smith, & Blake, 1998). Additional studies based on comparative analyses of DNA variation showed that *Ae. tauschii* accessions originating from the north eastern Fertile Crescent and its neighbouring regions of the Transcaucasus and the southern coastal Caspian Sea are genetically more similar to the D genome of bread wheat than those of other areas (Dvorak, Luo, Yang, & Zhang, 1998; Giles & Brown, 2006; Lelley, Stachel, Grausgruber, & Vollmann, 2000). More recently, a high-resolution genotype comparison between 10 wheat D-subgenomes and 242 *Ae. tauschii* accessions from across the distributional range revealed that the wheat D-subgenome consists of an admixture of distinct *Ae. tauschii* lineages; the accession BW_01028 (containing the rare *lr21-2* from Georgia and representing *Ae. tauschii* Lineage 3), contributed around 1.1% to the wheat D-subgenome (Gaurav et al., 2021). Multiple independent hybridization events between *T. turgidum* and *Ae. tauschii*, or gene-flow due to spontaneous outcrossing between hexaploid wheat and *Ae. tauschii* can explain the transfer of *lr21* alleles to bread wheat. However, more research is needed on the population genomics of stratified diversity sets of progenitor species and hexaploid wheat to bring this to a conclusion.

Next, we focused on the functional characterization of identified *Lr21* alleles for leaf rust resistance at the seedling and adult plant stage under field conditions. Seedling resistance was tested for pathogen isolate 77WxR which carries *avrLr21*. We found that the genotypes carrying *lr21-1* and *lr21-2* alleles showed similar susceptible responses, whereas genotypes carrying the *Lr21* allele revealed marginal differences in the resistance reaction ranging from small chlorotic spots to faint chlorosis or no visible infection. The fluctuation of resistance reactions in Syn022L and Tobak may be due to variations in the respective protein. Two amino acid substitution mutations were present in the NBS domain of the *Lr21-tbk* allele. The NBS domain of R proteins primarily mediates the oligomerization of proteins for triggering signal transduction cascades that lead to host immunity (van Wersch et al., 2020). Therefore, it is possible that variation in the NBS domain of the *Lr21-tbk* protein may underlie a mild resistance response in terms of a faint chlorosis in cultivar Tobak as compared to

Syn022L. In addition, a resistance reaction may depend on the genetic background; for the selected genotypes studied here, the disease symptoms in hexaploid backgrounds were consistently more pronounced both for susceptible and resistance reactions. The susceptibility was evident at the cellular level as high sporulation in genotypes carrying the *lr21-1* and *lr21-2* alleles, whereas the *Lr21* allele showed auto-fluorescence within the mesophyll cells around the infection sites in Syn022L. Such auto-fluorescence is related to the accumulation of phenolic compounds at early stages of infection which usually resulted in cessation of growth and the formation of chlorotic spots – a signature of resistance reaction at the point of infection (Bolton et al., 2008). These data suggest that the original *Lr21* allele from *Ae. tauschii* and a new *Lr21-tbk* from Tobak confer a resistance reaction against *P. triticina* isolate 77WxR leading to chlorosis and faint chlorosis at the seedling stage, respectively.

A further aspect of the *Lr21* function concerns the comparison of seedling and adult plant field leaf rust resistance in a population of 250 AB-lines. Comparative genetic mapping of seedling (inoculation with 77WxR) and adult plant field resistance (natural occurrence in multiple field environments) using high-resolution SNP-data identified *Lr21* to be associated with both resistances. These results led us to hypothesize a broader role of *Lr21* in conferring resistance against leaf rust beyond its previously known role for seedling resistance (Huang et al., 2009). To validate, we phenotyped an advanced backcross population segregating for leaf rust resistance in three consecutive years. We found a partial resistance phenotype in AB-lines carrying the *Lr21* resistance allele in contrast to obvious susceptibility in those harbouring the *lr21-2* allele. To evaluate *Lr21* regulation further, we quantified *Lr21* expression and found an abundance of *Lr21* transcripts at the seedling stage which decreased significantly later in the development in AB-line WW 22–75 (*Lr21*) and Batis (*lr21-2*). Higher and lower *Lr21* expressions were associated with a hypersensitive resistance reaction at the seedling stage and a partial resistance phenotype at the adult plant stage indicative of a putative role of expression dosage behind the observed functional variation. These data reveal a previously unknown role of *Lr21* in mediating partial resistance at the adult plant stage under field conditions. The presented data are primarily based on association analysis. Therefore, the involvement of additional genes or the role of genetic background effects in the above-mentioned resistance responses cannot be excluded. Previously, *Lr34* and *Lr67* were characterized as non-race specific adult plant resistance genes which encode an ATP-binding cassette (ABC) and a hexose transporter protein, respectively. These proteins confer broad-spectrum durable resistance against multiple pathogens. *Lr67* facilitates a non-race specific partial resistance due to the missing hexose transporter function leading to disturbance of a sugar balance in leaves. It was suggested that a reduced availability of nutrients within the host cell decreases the fungal growth on leaves (Krattinger et al., 2009; Moore et al., 2015). However, the basis of *Lr34*-mediated partial resistance was recently found to be linked with abscisic acid as a substrate of the ABC transporter (Krattinger et al., 2019). Unlike these resistances, our data suggest that *Lr21* confers adult plant slow rusting due to its development-dependent expressional variation. Such partial resistances can also be durable as it allows the co-existence of host and pathogen by maintaining the pathogen populations carrying the avirulence factors of *R* genes (Jones & Dangl, 2006). Avirulent pathogen populations are considered as important as *R* genes themselves in controlling resistance because of the functional dependency of *R* genes on pathogen effector proteins (Dangl & Jones, 2001). On the other hand, complete resistance presents a fast negative selection of avirulence factors from the pathogen population, thus such resistances are lost quickly. Taken together, these data suggest that unlike a complete resistance at seedling stage, *Lr21* confers a partial resistance against the natural occurrence of leaf rust at adult plant stages under field conditions.

In conclusion, the present study provides a first comprehensive insight into the population-wide evolutionary and functional divergence of *Lr21* in *Ae. tauschii* as compared to hexaploid wheat cultivars. The original *Lr21* allelic variants were successfully traced in wheat cultivars and functionally validated in both diploid and hexaploid backgrounds. Furthermore, the discovery of a unique functional *Lr21-tbk* allele in the hexaploid winter wheat cultivar Tobak conferring resistance to leaf rust was reported. In depths genetic analysis is needed to elucidate *Lr21-tbk* evolution and trace-back the sequence variation in the Tobak pedigree ([Elvis*Drifter]*Koch). Population genomics based on the *Lr21* sequence variation predicted at least two independent polyploidization events by which *lr21-1* and *lr21-2* alleles were transferred from *Ae. tauschii* to hexaploid wheat. Genetic erosion appeared to drive the negative selection of the primary functional *Lr21* allele in the evolution of hexaploid wheat. The transfer of the original *Lr21* allele into hexaploid wheat backgrounds conferred complete resistance at the seedling stage and a partial resistance under adult plant stage in field conditions. This new aspect of *Lr21* regulation provides an opportunity to survey additional *R* genes to fine-tune their expression in developing more durable resistances in wheat. In this regard, variation across the promoter of *R* genes can be valuable which can also be engineered precisely using genome-editing techniques such as CRISPR-Cas. Here, we propose that completely resistant hosts are undesirable for establishing long-term or broader resistances against complex rust pathogen populations. Therefore, future work should focus on the expressional differences of *R* genes and the dynamics of the pathogen population to maintain a co-existence of host and pathogen in terms of establishing a more durable resistance.

4 | MATERIALS AND METHODS

4.1 | Plant materials

An *Ae. tauschii* population comprising of 151 accessions were used in population genetics and candidate gene analyses. The details of this population, origin and geographic distribution were reported previously (Arora et al., 2019). In addition, hexaploid wheat cultivars Batis,

Zentos, Tobak and two synthetic accessions, Syn022L and Syn086L, were included. The development of the synthetic hexaploid wheat accessions Syn022L and Syn086L was based on chromosome-doubled hybrids (Lange & Jochemsen, 1992). For the genetic analyses of seedling and adult plant resistances, 250 BC2F3:6 lines of an advanced backcross population derived from crossing between the winter wheat cultivar Batis and the synthetic hexaploid wheat accession Sy022L were used following AB-QTL analysis strategy (Tanksley & Nelson, 1996). This population was designated as B22 and detailed information on its development can be found in previous reports (Kunert, Naz, Dedeck, Pillen, & Léon, 2007; Naz et al., 2008).

4.2 | Leaf rust seedling inoculations for leaf rust seedling resistance

Seedlings of three wheat (*Triticum aestivum* L.) genotypes (cvs. Batis, Monopol, Tobak) and of four *Aegilops tauschii* genotypes (BW_20624, BW_20644, BW_20983, BW_21416) were grown from seeds cultivated in plastic pots (9 × 9 × 8 cm^3) filled with seedling substrate (Klasmann-Deilmann GmbH, Geeste, Germany) and contained 4–10 seeds per pot. Each pot was covered by a cellophane bag under greenhouse conditions (22 ± 3°C, 16 hr illumination) for 2–3 weeks in order to avoid contamination with airborne pathogens. For inoculation experiments, leaf segments were produced from the second leaf at the three-leaf stage (BBCH 13). Leaf segments (6–7 cm) were placed on 2% water agar amended with 10 ppm benzimidazole. Each Petri dish included a leaf from all genotypes to be inoculated. In total, seven leaves (=7 genotypes) per Petri dish were inoculated with five replicates (Petri dishes); as the experiment was repeated, there were five biological replicates and two technical replicates for the detached leaf assay.

4.2.1 | Leaf rust inoculation and incubation for microscopic evaluation

Spores of *P. triticina* isolate 77WxR (*avrLr21*) were propagated on wheat cv. Monopol having no (known) resistance to *P. triticina*. Urediniospores harvested from mature uredia were stored at 6–8°C in 1.5 ml reaction tubes. The leaves of Batis, Zentos, Syn022L and Syn086L in Petri dishes were spray-inoculated with urediniospores (75–100 mg) suspended in 150 ml water containing one droplet of Tween 80 as detergent avoiding run-off (leaf segment tests or detached leaf assay) (Serfling, Templer, Winter, & Ordon, 2016). Plants were inoculated at the three-leaf stage by spraying the same spore suspension until run-off (attached leaf assay). After inoculation, the dishes with inoculated leaf segments were sealed with parafilm and were incubated at 22 ± 1°C, 16 hr illumination until the development of typical infection types. The plants were kept at 100% relative humidity for 24 hr, subsequently at 60–70% in the growth chamber with a 16 hr/8 hr light/dark and a temperature of 20–22°C. From these plants, segments of the oldest leaf with a length of 1 cm were taken for microscopy. Disease severity was assessed by counting the number of uredinia per leaf segment (detached leaves) and by estimating the percentage leaf area covered by uredinia (attached leaves). The size of uredinia was assessed as the area per uredinium by using a Leica MZ16 F stereo microscope (Leica, Wetzlar, Germany) with a mounted digital camera KY-F75U (JVC, Yokohama, Japan) and the Diskus software (Fa. Hilgers, Königswinter, Germany). In order to analyse defence reactions and fungal structures, microscopical analysis of host-pathogen interactions was performed according to Serfling et al. (2016) by a calcofluor stain 72 and 168 hr after inoculation. Defence reactions were recorded by autofluorescence, visible within plant tissue, using the filter set 05 (excitation filter BP 400–440, beam splitter FT 460, barrier filter LP 470).

4.2.2 | Disease assessment for leaf rust seedling resistance

For seedling stage resistance, the population B22 was sown in 96-cell trays in two randomized complete blocks with individual lines replicated three times each block as described in Naz et al. (2008). Both parents were included and replicated five times per block as controls. Initially, the susceptible cultivar Borenos was inoculated with *P. triticina* pathotype 77WxR with the help of a mechanical duster. Later, the fresh spores from the pre-infected seedlings of Borenos were trickled onto two-week-old seedlings of population B22. Scoring of leaf rust symptoms was carried out 10 days after inoculation. Infection types (IT) of *P. triticina* on the genotypes were classified and quantified based on a standard 0–4 scale (Long & Kolmer, 1989). According to the scale, 0 = immune; = hypersensitive fleck without uredinia, 1 = small uredinia surrounded by necrosis, 2 = small uredinia surrounded by chlorosis, 3 = moderate size uredinia that may be associated with chlorosis, 4 = large uredinia without chlorosis.

4.3 | Population genomics and phylogenetic analyses

Sequence of the *Lr21* gene from 145 *Ae. tauschii* accessions were used to describe the *Lr21* alleles among the genotypes. To distinguish ancestral clusters in the population K means clustering was performed in R. For the *Lr21* allele, the first three principal components were calculated and plotted by using function *bpca* (*PCAMethods)*. The Q-matrix was generated by applying the functions from the *R package tess3R*, and subsequently used in the creation of the ancestral proportion. Finally, 151 genotypes were compiled on a geographic map based on available longitude and latitude information. A plot was created by incorporating the information of the Q matrix and the *Lr21* allele to generate a link on all information layers. Lastly, a phylogenetic tree was created based on the genotyping data by *hclust function* in R with the method *ward.D2* following *euclidean* distance. Another phylogenetic tree was created for the *Lr21* haplotypes. For this, *Lr21* allele sequences from 145 genotypes, including the synthetic genotype Syn022L, Chinese Spring, Batis and Tobak were utilized. The

sequences were aligned by *MEGA* alignment (Version 10.0.5), using the *muscle* algorithm with default settings. The reference *Lr21* allele (Syn022L) was used to trim excessive base pairs from the sequencing alignment of the genotypes. Followed by this, the variants and haplotypes of the genotypes were identified and visualized in another phylogenetic tree with the same procedure as described before. A total of 100 bootstraps were performed to test the precision of differentiation between the genotypes.

4.4 | DNA extraction and gene sequencing

DNA was purified using a DNA extraction protocol for wheat and barley according to reported protocol (Yu, Hatta, Periyannan, Lagudah, & Wulff, 2017). The protocol was optimized for 96-well plates. Leaf material was collected from 2 weeks old seedlings of each genotype. Samples were stored overnight at −80°C and then ground with a TissueLyser (Qiagen, Hilden, Germany), with the settings of 25 Hz, two times 60 s. A total of 300 μl CTAB buffer (1 M Tris, 5 M NaCl, 0.25 M EDTA, CTAB, H_2O) plus 1 μl RNase (10 mg/μl) was added to the samples and incubated at 65°C for 30 min. Later, 400 μl Chloroform/ Isoamylalcohol (24:1) was added and inverted several times with additional shaking of 1 hr at 600 rpm. Afterwards, the samples were centrifuged for 20 min at 6,000 rpm. The supernatant (around 300 μl) was transferred to new 96-well tubes and 300 μl ice cold isopropanol was added. The plates were inverted and stored overnight at 4°C. The samples were centrifuged for 20 min at 6,000 rpm. The supernatant was discarded and the pellets were washed by adding two times 500 μl 75% ethanol, respectively. After each washing step, the samples were centrifuged for 20 min at 6,000 rpm. The pellet was air-dried until the ethanol had evaporated and then re-suspended in 100 μl DNase free water, and then kept for 1 hr at room temperature to dry. DNA samples were kept at 4°C for short-term storage and at −20°C for long-term storage.

Sequencing of Lr21 in candidate genotypes were performed using Sanger sequencing method. PCR and sequencing primers information are given in Table S2. In addition, a global diversity set of 89 wheat cultivars was sequenced for the *Lr21* causal mutations for the haplotypes analysis and for allele mining.

4.5 | *Lr21* mRNA expression analysis

Lr21 expression was evaluated 2, 4, 8 and 12 weeks after sowing. For this, the recurrent parent Batis and the advanced backcross line WW22-75 carrying the *Lr21* allele derived from Syn022L were used in two biological replicates at first and later on in three replicates for each time-point. For RNA extraction 2 cm long leaf sections were collected, directly frozen in liquid nitrogen and stored at −80°C. Samples were ground in liquid nitrogen and 800 μl Z6 buffer (4 M guanidine thiocyanate, 20 mM EDTA, 20 mM MES, 0.7% [v/v] β-mercaptoethanol) was added to each sample. The samples were incubated for 10 min at room temperature, and 300 μl phenol: chloroform: isoamylalcohol (25:24:1) was added and vortexed. Next samples were centrifuged at 12,000 rpm at 4°C for 15 min. The supernatant was transferred to new RNase free tubes and 40 μl CH_3COOH plus 560 μl absolute ethanol was added to each sample. The samples were incubated overnight at −20°C. Afterwards, the samples were centrifuged at 12,000 rpm at 4°C for 20 min. The supernatant was discarded and the pellet was washed twice with 500 μl 80% ethanol. Each time, the samples were centrifuged at 12,000 rpm at 4°C for 5 min. The pellet was air-dried for 5–10 min and re-suspended in 40 μl RNase free water for 1 hr at room temperature. The DNase purification step of RNA was done using Ambion DNase-free kit (Invitrogen, Carlsbad, California), following the manufacturer protocol. Samples were incubated for 30 min at 37°C. One-tenth part of the total volume DNase inactivation reagent was added and the samples were then incubated for 2 min at room temperature followed by centrifugation for 1.5 min at 10,000 rpm. The RNA was transferred to a new DNA free tube avoiding touching of the pellets. For short-term storages, the samples were kept at −20°C, while for long-term storage they were kept at −80°C. For reverse transcription of RNA, FirstStrand cDNA synthesis kit from Thermo Fisher Scientific (Waltham, Massachusetts) was used following the manufacturer protocol. A qPCR was conducted using three technical replicates of each biological replicate at each time-point on an Applied Biosystems 7,500 Fast Real-Time PCR cycler (Applied Biosystems, Waltham, Massachusetts). Actin was used as an internal housekeeping control gene. Primer information for actin and *Lr21* genes can be found in Table S2.

4.6 | Adult plant field resistance evaluations

The evaluation of field resistance in the population B22 was carried out under natural infection. Initially the population B22 was evaluated (field evaluation I) in seasons 2004 (04) and 2005 (05) at different environments across Germany. The experimental stations were Dikopshof, University of Bonn (D04 and D05), Rosenthal, Limagrain-Nickerson GmbH (R05) and Söllingen, Fr. Strube Saatzucht KG (S04 and S05). In each environment, 250 AB-lines and the recurrent parent Batis were evaluated in a randomized complete block design, comprising of one plot of each AB-line and eight replications of the recurrent parent Batis. The plots were treated with NPK fertilizer according to local practice and no fungicides were applied. Later, the validation experiments for field resistance (field evaluation II) under natural infection of leaf rust in population B22 were carried out in three consecutive years (2014/15–2016/17) and in three environments located in Wetze (KWS Lochow), Rosenthal (Limagrain) and Aspachhof (Saatzucht Streng-Engelen). Here, the setup was a randomized block design, where 250 B22 genotypes were sown in one square meter plots (one replication each). Additional to the AB lines, eight cultivars (Batis, Capone, Colonia, Dekan, Elixer, Genius, Pionier and Rumor) were evaluated in five replications as control genotypes for resistance/susceptibility to leaf rust. Fertilizers were applied following conventional breeder standards. Phenotyping was carried out by visual

scoring of leaf rust, using the scoring system of Bundessortenamt (Hannover, Germany) from 1 (resistant) to 9 (susceptible).

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CONFLICT OF INTERESTS

Authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Ali A. Naz, Brande B. H. Wulff, Klaus Pillen, Frank Ordon and Jens Léon conceptualized the research. Annemarie Bungartz, Albrecht Serfling, Ali A. Naz, Mohammad Kamruzzaman, Michael Schneider, Agim Ballvora and Erich-Christian Oerke performed the laboratory and field experiments. Ali A. Naz, Annemarie Bungartz, Albrecht Serfling, Erich-Christian Oerke, Mohammad Kamruzzaman and Michael Schneider performed data analysis. Ali A. Naz, Albrecht Serfling, Annemarie Bungartz, Klaus Pillen, Jens Léon, Brande B. H. Wulff, Agim Ballvora, Erich-Christian Oerke and Frank Ordon wrote the manuscript.

DATA AVAILABILITY STATEMENT

The genotype data of the population *Ae. tauschii* was previously published by Arora et al. (2019). The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures and tables.

REFERENCES

Arora, S., Cheema, J., Poland, J., Uauy, C., & Chhuneja, P. (2019). Genome-wide association mapping of grain micronutrients concentration in *Aegilops tauschii*. *Frontiers in Plant Science*, *10*, 54.

Bolton, M. D., Kolmer, J. A., & Garvin, D. F. (2008). Wheat leaf rust caused by Puccinia triticina. *Molecular Plant Pathology*, *9*(5), 563–575. https://pubmed.ncbi.nlm.nih.gov/19018988/

Bonardi, V., Tang, S., Stallmann, A., Roberts, M., Cherkis, K., & Dangl, J. L. (2011). Expanded functions for a family of plant intracellular immune receptors beyond specific recognition of pathogen effectors. *Proceedings of the National Academy of Sciences of the United States of America*, *108*, 16463–16468.

Boyd, L. A., Ridout, C., O'Sullivan, D. M., Leach, J. E., & Leung, H. (2013). Plant-pathogen interactions: Disease resistance in modern agriculture. *Trends in Genetics*, *29*, 233–240.

Cesari, S. (2018). Multiple strategies for pathogen perception by plant immune receptors. *The New Phytologist*, *219*, 17–24.

Collier, S. M., Hamel, L.-P., & Moffett, P. (2011). Cell death mediated by the N-terminal domains of a unique and highly conserved class of NB-LRR protein. *Molecular Plant-Microbe Interactions*, *24*, 918–931.

Dangl, J. L., & Jones, J. D. G. (2001). Plant pathogens and integrated defence responses to infection. *Nature*, *411*, 826–833.

DeYoung, B. J., & Innes, R. W. (2006). Plant NBS-LRR proteins in pathogen sensing and host defense. *Nature Immunology*, *7*, 1243–1249.

Dilbirligi, M., Erayman, M., Sandhu, D., Sidhu, D., & Gill, K. S. (2004). Identification of wheat chromosomal regions containing expressed resistance genes. *Genetics*, *166*, 461–481.

Dvorak, J., Luo, M. C., Yang, Z. L., & Zhang, H. B. (1998). The structure of the *Aegilops tauschii* genepool and the evolution of hexaploid wheat. *Theoretical and Applied Genetics*, *97*, 657–670.

Ellis, J. G., Lawrence, G. J., Luck, J. E., & Dodds, P. N. (1999). Identification of regions in alleles of the flax rust resistance gene L that determine differences in gene-for-gene specificity. *Plant Cell*, *11*, 495–506.

Flor, H. H. (1942). Inheritance of pathogenicity in *Melampsora lini*. *Phytopathology*, *32*, 653–669.

Gaurav, K., Arora, S., Silva, P., Sánchez-Martín, J., Horsnell, R., Gao, L. & Wulff, B. B. H. … (2021). Evolution of the bread wheat D-subgenome and enriching it with diversity from Aegilops tauschii. bioRxiv January 31, 2021.428788. https://doi.org/10.1101/2021.01.31.428788

German, S. E., & Kolmer, J. A. (1992). Effect of gene Lr34 in the enhancement of resistance to leaf rust of wheat. *Theoretical and Applied Genetics*, *84*, 97–105.

Giannakopoulou, A., Steele, J. F. C., Segretin, M. E., Bozkurt, T. O., Zhou, J., Robatzek, S., … Kamoun, S. (2015). Tomato I2 immune receptor can be engineered to confer partial resistance to the Oomycete *Phytophthora infestans* in addition to the fungus *Fusarium oxysporum*. *Molecular Plant-Microbe Interactions*, *28*, 1316–1329.

Giles, R. J., & Brown, T. A. (2006). GluDy allele variations in Aegilops tauschii and *Triticum aestivum*: Implications for the origins of hexaploid wheats. *Theoretical and Applied Genetics*, *112*, 1563–1572.

Huang, L., Brooks, S., Li, W., Fellers, J., Nelson, J. C., & Gill, B. (2009). Evolution of new disease specificity at a simple resistance locus in a crop-weed complex: Reconstitution of the Lr21 gene in wheat. *Genetics*, *182*, 595–602.

Huang, L., Brooks, S. A., Li, W., Fellers, J. P., Trick, H. N., & Gill, B. S. (2003). Map-based cloning of leaf rust resistance gene Lr21 from the large and polyploid genome of bread wheat. *Genetics*, *164*, 655–664.

Jones, J. D. G., & Dangl, J. L. (2006). The plant immune system. *Nature*, *444*, 323–329.

Jubic, L. M., Saile, S., Furzer, O. J., El Kasmi, F., & Dangl, J. L. (2019). Help wanted: Helper NLRs and plant immune responses this review comes from a themed issue on biotic interactions. *Current Opinion in Plant Biology*, *50*, 82–94.

Kolmer, J. A. (2005). Tracking wheat rust on a continental scale. *Current Opinion in Plant Biology*, *8*, 441–449.

Kolmer, J. A., Singh, R. P., Garvin, D. F., Viccars, L., William, H. M., Huerta-Espino, J., … Lagudah, E. S. (2008). Analysis of the Lr34/Yr18 Rust Resistance Region in Wheat Germplasm. *Crop Science*, *48*, 1841–1852. https://acsess.onlinelibrary.wiley.com/doi/full/10.2135/cropsci2007.08.0474

Kourelis, J., & Van Der Hoorn, R. A. L. (2018). Defended to the nines: 25 years of resistance gene cloning identifies nine mechanisms for R protein function. *Plant Cell*, *30*, 285–299.

Krattinger, S. G., Kang, J., Bräunlich, S., Boni, R., Chauhan, H., Selter, L. L., … Keller, B. (2019). Abscisic acid is a substrate of the ABC transporter encoded by the durable wheat disease resistance gene *Lr34*. *The New Phytologist*, *223*, 853–866.

Krattinger, S. G., Lagudah, E. S., Spielmeyer, W., Singh, R. P., Huerta-Espino, J., McFadden, H., … Keller, B. (2009). A putative ABC transporter confers durable resistance to multiple fungal pathogens in wheat. *Science*, *323*, 1360–1363.

Kumar, S., Bhardwaj, S. C., Gangwar, O. P., Sharma, A., Qureshi, N., Kumaran, V. V., … Bansal, U. K. (2021). Lr80: A new and widely

effective source of leaf rust resistance of wheat for enhancing diversity of resistance among modern cultivars. *Theoretical and Applied Genetics*, *134*, 849–858.

Kunert, A., Naz, A. A., Dedeck, O., Pillen, K., & Léon, J. (2007). AB-QTL analysis in winter wheat: I. Synthetic hexaploid wheat (T. turgidumssp. dicoccoides·T. tauschii) as a sourceof favourable alleles for milling and baking quality traits. *Theoretical and Applied Genetics*, *115*, 683–695.

Lange, W., & Jochemsen, G. (1992). Use of the gene pools of Triticum turgidum ssp. dicoccoides and *Aegilops squarrosa* for the breeding of common wheat (*T. aestivum*), through chromosome-doubled hybrids - II. Morphology and meiosis of the amphiploids. *Euphytica*, *59*, 213–220.

Lelley, T., Stachel, M., Grausgruber, H., & Vollmann, J. (2000). Analysis of relationships between *Aegilops tauschii* and the D genome of wheat utilizing microsatellites. *Genome*, *43*, 661–668.

Long, D. L., & Kolmer, J. A. A. (1989). North American system of nomenclature for Puccinia recondita f. sp. tritici. *Phytopathology*, *79*, 525–529.

Meyers, B. C., Kozik, A., Griego, A., Kuang, H., & Michelmore, R. W. (2003). Genome-wide analysis of NBS-LRR-encoding genes in Arabidopsis. *Plant Cell*, *15*, 809–834.

Michelmore, R. W., & Meyers, B. C. (1998). Clusters of resistance genes in plants evolve by divergent selection and a birth-and-death process. *Genome Research*, *8*, 1113–1130.

Moore, J. W., Herrera-Foessel, S., Lan, C., Schnippenkoetter, W., Ayliffe, M., Huerta-Espino, J., … Lagudah, E. (2015). A recently evolved hexose transporter variant confers resistance to multiple pathogens in wheat. *Nature Genetics*, *47*, 1494–1498.

Naz, A. A., Kunert, A., Lind, V., Pillen, K., & Léon, J. (2008). AB-QTL analysis in winter wheat: II. Genetic analysis of seedling and field resistance against leaf rust in a wheat advanced backcross population. *Theoretical and Applied Genetics*, *116*, 1095–1104.

Pellinen, R. I., Korhonen, M.-S., Tauriainen, A. A., Palva, E. T., & Kangasjärvi, J. (2002). Hydrogen peroxide activates cell death and defense gene expression in birch. *Plant Physiology*, *130*, 549–560. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC166586/

Risk, J. M., Selter, L. L., Krattinger, S. G., Viccars, L. A., Richardson, T. M., Buesing, G., … Keller, B. (2012). Functional variability of the Lr34 durable resistance gene in transgenic wheat. *Plant Biotechnology Journal*, *10*, 477–487.

Rowland, G. G., & Kerber, E. R. (1974). Telocentric mapping in HEXAPLOID wheat of genes for LEAF rust resistance and other characters derived from *AEGILOPS SQUARROSA*. *Canadian Journal of Genetics and Cytology*, *16*, 137–144.

Savary, S., Willocquet, L., Pethybridge, S. J., Esker, P., McRoberts, N., & Nelson, A. (2019). The global burden of pathogens and pests on major food crops. *Nature Ecology & Evolution*, *3*, 430–439.

Serfling, A., Templer, S. E., Winter, P., & Ordon, F. (2016). Microscopic and molecular characterization of the prehaustorial resistance against wheat leaf rust (Puccinia triticina) in einkorn (*Triticum monococcum*). *Frontiers in Plant Science*, *7*, 1668.

Shan, W. X., Cao, M., Dan, L. U., & Tyler, B. M. (2004). The Avr1b locus of Phytophthora sojae encodes an elicitor and a regulator required for avirulence on soybean plants carrying resistance gene Rps1b. *Molecular Plant-Microbe Interactions*, *17*, 394–403.

Singla, J., Lüthi, L., Wicker, T., Bansal, U., Krattinger, S. G., & Keller, B. (2017). Characterization of Lr75: A partial, broad-spectrum leaf rust resistance gene in wheat. *Theoretical and Applied Genetics*, *130*, 1–12.

Steuernagel, B., Witek, K., Krattinger, S. G., Ramirez-Gonzalez, R. H., Schoonbeek, H. J., Yu, G., … Wulff, B. B. H. (2020). The NLR-annotator tool enables annotation of the intracellular immune receptor repertoire. *Plant Physiology*, *183*, 468–482.

Talbert, L. E., Smith, L. Y., & Blake, N. K. (1998). More than one origin of hexaploid wheat is indicated by sequence comparison of low-copy DNA. *Genome*, *41*, 402–407.

Tanksley, S. D., & Nelson, J. C. (1996). Advanced backcross QTL analysis: A method for the simultaneous discovery and transfer of valuable QTLs from unadapted germplasm into elite breeding lines. *Theoretical and Applied Genetics*, *92*, 191–203.

Van Kan, J. A. L., Van Den Ackerveken, G. F. J. M., & De Wit, P. J. G. M. (1991). Cloning and characterization of cDNA of avirulence gene avr9 of the fungal pathogen Cladosporium fulvum, causal agent of tomato leaf mold. *Molecular Plant-Microbe Interactions*, *4*, 52–59.

van Wersch, S., Tian, L., Hoy, R., & Li, X. (2020). Plant NLRs: The whistleblowers of plant immunity. *Plant Communications*, *1*, 100016.

Yu, G., Hatta, A., Periyannan, S., Lagudah, E., & Wulff, B. B. H. (2017). Isolation of wheat genomic DNA for gene mapping and cloning. *Methods in Molecular Biology*, *1659*, 207–213.

SUPPORTING INFORMATION

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

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