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ORIGINAL ARTICLE

Molecular Plant Pathology WILEY

Remarkable recent changes in the genetic diversity of the avirulence gene *AvrStb6* in global populations of the wheat pathogen *Zymoseptoria tritici*

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Abstract

Septoria tritici blotch (STB), caused by the fungus *Zymoseptoria tritici*, is one of the most economically important diseases of wheat. Recently, both factors of a gene-for-gene interaction between *Z. tritici* and wheat, the wheat receptor-like kinase Stb6 and the *Z. tritici* secreted effector protein AvrStb6, have been identified. Previous analyses revealed a high diversity of *AvrStb6* haplotypes present in earlier *Z. tritici* isolate collections, with up to c.18% of analysed isolates possessing the avirulence isoform of AvrStb6 identical to that originally identified in the reference isolate IPO323. With *Stb6* present in many commercial wheat cultivars globally, we aimed to assess potential changes in *AvrStb6* genetic diversity and the incidence of haplotypes allowing evasion of *Stb6*-mediated resistance in more recent *Z. tritici* populations. Here we show, using targeted resequencing of *AvrStb6*, that this gene is universally present in field isolates sampled from major wheat-growing regions of the world in 2013–2017. However, in contrast to the data from previous *AvrStb6* population studies, we report a complete absence of the originally described avirulence isoform of AvrStb6 amongst modern *Z. tritici* isolates. Moreover, a remarkably small number of haplotypes, each encoding AvrStb6 protein isoforms conditioning virulence on *Stb6*-containing wheat, were found to predominate among modern *Z. tritici* isolates. A single virulence isoform of AvrStb6 was found to be particularly abundant throughout the global population. These findings indicate that, despite the ability of *Z. tritici* to sexually reproduce on resistant hosts, *AvrStb6* avirulence haplotypes tend to be eliminated in subsequent populations.

KEYWORDS
disease resistance, fungal effector, population biology, Septoria tritici blotch, *Stb6*, *Triticum aestivum*

1 | INTRODUCTION

The interactions between plant pathogens and their hosts during infection are highly complex and evolutionarily dynamic. Effectors, molecules including proteins that are produced and secreted by pathogens during infection, constitute a vital part of the repertoire of mechanisms utilized in the successful infection of plant hosts (Lo Presti et al., 2015). Effectors function by altering the metabolism of the host plant to facilitate infection or by suppressing plant immune responses to infection (Djamei et al., 2011; Kleemann et al., 2012; Marshall et al., 2011). However, plants possess a sophisticated innate immune system whose central players are cell surface and intracellular immune receptors including disease resistance (*R*) genes, which are capable of detecting pathogen effectors and initiating an immune response (Cook et al., 2015; Jones & Dangl, 2006; Kanyuka & Rudd, 2019).

Recognition of a pathogen effector protein by a plant immune receptor thereby introduces an evolutionary pressure on the pathogen to mutate or lose the recognized effector (otherwise known as an avirulence or Avr factor) entirely from its genome. Avr factors may be lost through frameshift or nonsense mutations (Luderer et al., 2002), transposon insertion (Zhang et al., 2015), or repeat-induced point mutations (RIPs) (Rouxel et al., 2011). Indeed, effectors are often located in genomic regions rich in transposon activity, which drives effector diversity (Dong et al., 2015). Suppression of Avr factor expression, through mutations in the promoter, histone modification (Wang et al., 2020), or posttranscriptional silencing (Qutob et al., 2013), may also restore pathogen virulence. In some cases, point mutations in the *Avr* gene sequence may allow evasion of recognition whilst maintaining protein function (Blondeau et al., 2015; Plissonneau et al., 2017). This may happen in cases where the Avr factor is important for pathogen fitness. Once mutations in *Avr* genes have occurred that allow evasion of detection by immune receptors, they often spread rapidly through the pathogen population, particularly when the cognate disease resistance gene is widely used (Cowger et al., 2000; Hovmøller & Justesen, 2007).

Zymoseptoria tritici, the causal agent of Septoria tritici blotch (STB), is one of the most economically important fungal pathogens of wheat, with fungicide control costs exceeding €1 billion per year in Europe alone (Torriani et al., 2015). *Z. tritici* secretes an array of putative effectors during the infection cycle, among which is the avirulence factor AvrStb6 (Kema et al., 2018; Zhong et al., 2017), recognized in a gene-for-gene manner by the corresponding cell surface immune receptor Stb6 (Brading et al., 2002; Saintenac et al., 2018). *AvrStb6* has been identified in the genomes of the isolates IPO323 and 1E4 collected from the Netherlands and Switzerland in 1981 and 1999, respectively, and recently cloned (Kema et al., 2018; Zhong et al., 2017). The *Stb6* gene has also been recently cloned from wheat landrace Chinese Spring and the UK cultivar (cv.) Cadenza (Saintenac et al., 2018). *AvrStb6* and *Stb6* encode a small, cysteine-rich secreted protein and a wall-associated receptor-like kinase protein, respectively. A functional haplotype of *Stb6* conferring resistance to *Z. tritici* IPO323 is found in over half of commercial cultivars in Europe (Saintenac et al., 2018) and may have been present in wheat populations globally since early in agricultural history (Chartrain et al., 2005). Consequently, pressure on *Z. tritici* to lose *AvrStb6* variants conditioning avirulence on *Stb6*-containing wheat must be considerable.

There are two previous studies in which sequencing of *AvrStb6* from populations of *Z. tritici* has been carried out. In one study, a global population of *Z. tritici* collected between 1990 and 2001 (Brunner & McDonald, 2018; Zhan et al., 2005) was analysed, whilst the second focused on a largely French population collected in 2009–2010 (Zhong et al., 2017). These studies found a high diversity of *AvrStb6* haplotypes and evidence of positive selection driven by point mutations and recombination. The region of *Z. tritici* chromosome 5 in which *AvrStb6* is located was found to be highly dynamic, with extensive transposon activity contributing to *AvrStb6* polymorphism (Sánchez-Vallet et al., 2018).

Whilst the *AvrStb6* haplotype distribution in these earlier collections has been well characterized, the *AvrStb6* haplotype diversity in more modern *Z. tritici* populations is unknown. Also unknown is the prevalence of *Z. tritici* isolates that possess *AvrStb6* haplotypes capable of evading *Stb6*-mediated defence. The specific polymorphisms that drive the change from avirulence to virulence phenotype in the AvrStb6 protein have not yet been determined, although changes at two amino acid residues (positions 41 and 43) in the AvrStb6 protein have been suggested as being critical for the pathogenicity on wheat cultivars carrying *Stb6* (Kema et al., 2018).

In this study, we resequenced the *AvrStb6* gene from recent field populations of *Z. tritici* isolates collected between 2013 and 2017, therefore providing a global snapshot of *AvrStb6* haplotype diversity at this time. We show a notable decrease in the haplotype diversity of *AvrStb6* compared to previous studies, including a recent worldwide shift towards haplotypes encoding *Stb6* resistance-breaking isoforms. Interestingly, one particular virulence isoform (I02) was found to be the most abundant in several regions of the world examined. This study therefore provides a rare insight into temporal changes in pathogen effector diversity in response to the host-imposed pressures and highlights the population shifts that can occur when a single corresponding disease resistance gene is deployed extensively.

2 | RESULTS

2.1 | *AvrStb6* haplotype analysis

Primers flanking the *AvrStb6* gene were designed and used for PCR amplification of this gene from a collection of 383 *Z. tritici* isolates sampled from different field-grown bread wheat (*Triticum aestivum*) cultivars in 10 countries on five continents. PCR products of expected size were obtained from all analysed samples, indicating *AvrStb6* is present in all isolates in the collection. Sequencing of the PCR products revealed a total of 52 *AvrStb6* haplotypes (denoted from H01 through to H52; Table S1) coding for 44 protein isoforms (denoted from I01 through to I44; Figure 1). This includes *AvrStb6* haplotypes and the corresponding protein isoforms from
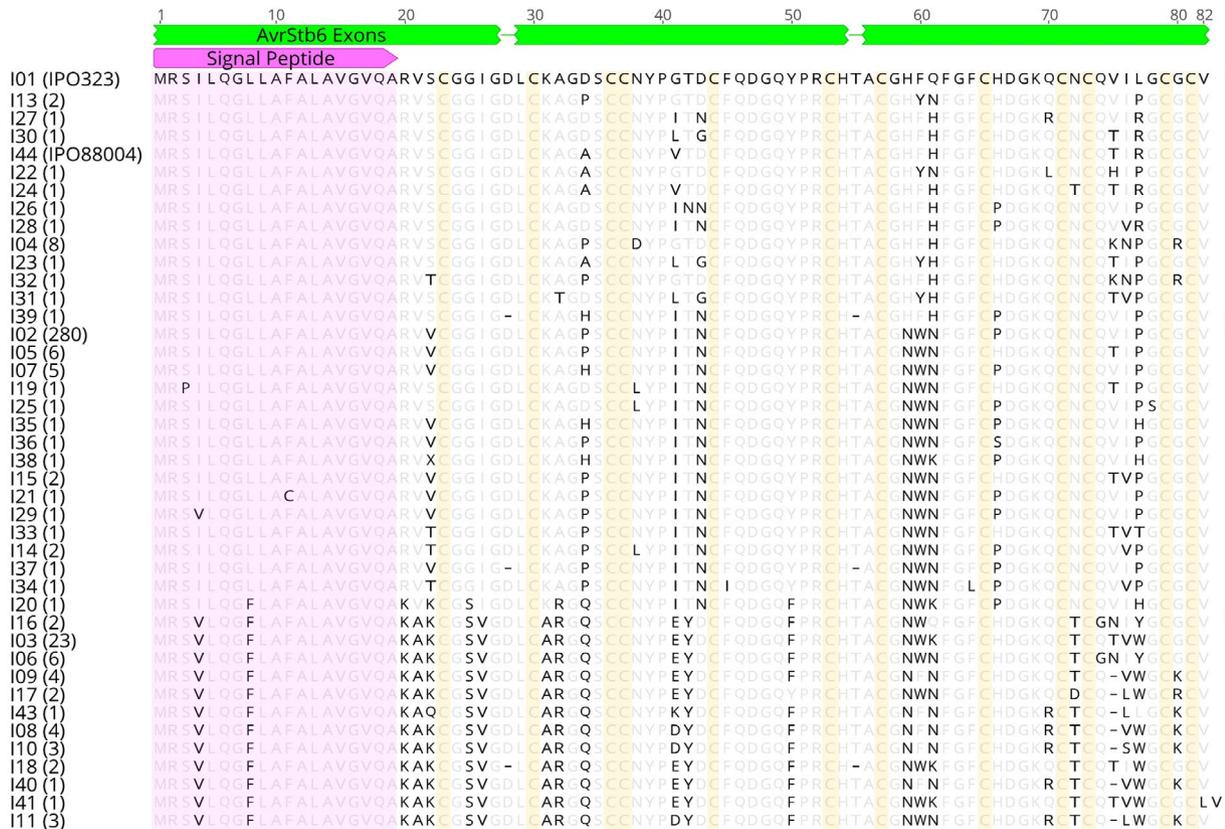
FIGURE 1 Alignment of the AvrStb6 isoform sequences identified in this study. Protein sequences were aligned using MAFFT v. 7.388. Numbers in parentheses represent the number of isolates identified possessing each isoform. Isoforms I01 and I44 have been identified in the historic *Zymoseptoria tritici* isolates IPO323 and IPO88004, which are avirulent or virulent on *Stb6* wheat, respectively. Amino acids synonymous to the I01 sequence are greyed. Missing residues relative to I01 are represented as dashes. The pink arrow annotates the secretion signal peptide, whilst the green bars represent the exons of the coding gene sequence. The three alignment groupings (I, II, and III) of the isoforms are indicated by vertical blue lines to the right of the sequences, and invariant cysteine residues are highlighted in yellow

the reference isolate IPO323 (H01/I01) and another well-studied isolate IPO88004 (H52/I44), which are avirulent or virulent on *Stb6*-containing wheat, respectively.

Almost all haplotypes were predicted to encode full-length AvrStb6 protein. The exceptions to this were H17 (only present in four isolates from Turkey) containing a nonsense mutation in the exon 1, and H24 and H46 (each represented by a single isolate also from Turkey) that contained single nucleotide deletions/frameshift mutations located in exon 3 and exon 2, respectively. The majority of recovered *AvrStb6* sequences (361 of 381) were 365 bp in length from the ATG start codon to the TGA stop codon and including two introns, with others ranging in lengths from 362 to 366 bp. Most of the nucleotide polymorphisms were in exons 2 and 3, with exon 1 being more conserved, and particularly within the sequence coding for the N-terminal signal peptide (Figure 1). Eight haplotypes (H09, H10, H12, H21, H22, H23, H37, and H51) represented by 18 isolates contained three types of in-frame 3-bp deletions in exon 3. These haplotypes collapse to seven protein isoforms (I08, I09, I10, I11, I17, I40, and I43). All these isoforms apart from I17, represented by two isolates from western Europe, were exclusively found among the *Z. tritici* isolates from Turkey. The identified AvrStb6 isoforms were composed of 80–82 amino acid residues. From these, residues

at 52 positions, including all 12 cysteines, were invariant across the collection (excluding the I42 frameshift mutant) (Figure 1). All other amino acid residues in AvrStb6 were variable, with the highest variation identified at positions 41, 61, and 77. As revealed by the amino acid sequence alignment and haplotype network analysis, AvrStb6 isoforms fell generally into three groups denoted I, II, and III (Figures 1, 2a, and S1). This, in principle, was supported by a phylogenetic analysis, which also identified that group III haplotypes, largely represented by isolates of Turkish origin, are phylogenetically more distant from those in groups I and II (Figure 2b).

2.2 | One AvrStb6 isoform predominates among current *Z. tritici* isolates globally

By far the most prevalent AvrStb6 isoform in the collection was I02, identified in 280 of 381 isolates (73.5%). This isoform was the most common in almost every geographic region studied, including Australia (96.6%), western Europe (91.8%), the United States (82.7%), and Chile (80%) (Table 1). The only exceptions were Argentina and Uruguay, where isoform I03 was most prevalent, and Turkey, where isoform I02 was not identified at all. AvrStb6 isoform I02 was found to be encoded
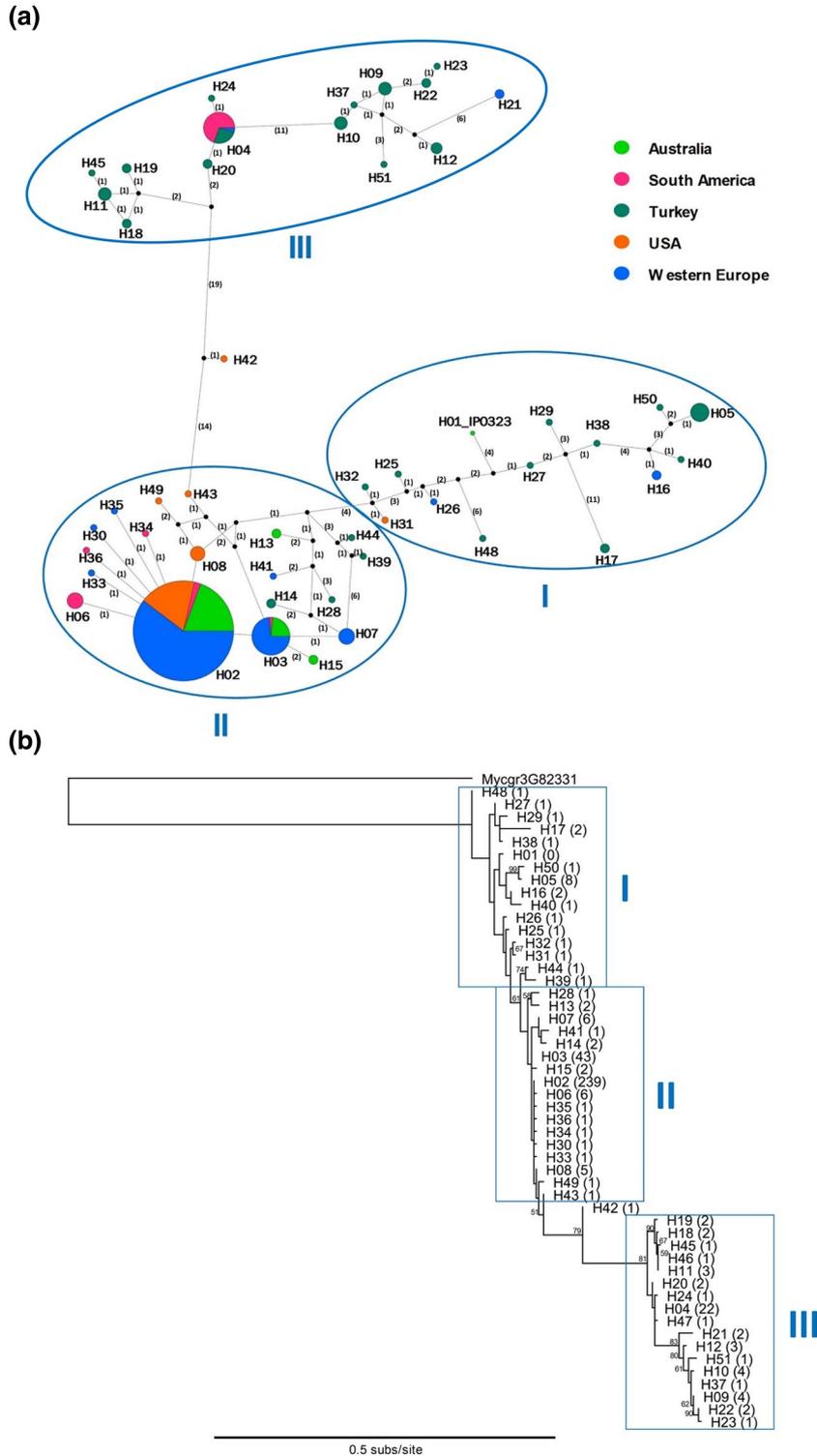
(a)

(b)

FIGURE 2 Analysis of the *AvrStb6* haplotypes distribution in the global *Zymoseptoria tritici* population. (a) Haplotype network for global *AvrStb6* sequences obtained from the TCS analysis. Each node represents a separate haplotype identified in this study. The node size represents the number of isolates identified possessing the same haplotype. The nodes are painted in different colours depending on the geographic region(s) from which the isolates of the same node were identified. Connecting lines between nodes denote closely related haplotypes, with numbers in parentheses corresponding to the number of mutations between adjoined haplotypes, but line lengths are not proportional to genetic distance. Black unlabelled nodes represent hypothetical common ancestors of related haplotypes. The three groupings of haplotypes I, II, and III are highlighted by blue ellipses. (b) Phylogenetic tree of the *AvrStb6* haplotypes constructed using PhyML. The tree was rooted using the *Z. tritici* gene *Mycgr3G82331* located on chromosome 10, a paralogue of *AvrStb6*. Numbers on branches indicate bootstrap scores for each branch, whilst numbers in parentheses indicate the number of isolates identified possessing each haplotype

by several haplotypes: H02 (239 isolates), H03 (34 isolates), H06 (6 isolates), H15 (2 isolates), and H33 (1 isolate). However, in the US collection, only a single haplotype (H02) was found to code for I02.

The next most common isoform globally was I03, the only other isoform to be found in multiple regions, which was identified in 23 isolates (6% of the isolate collection) in western Europe, South America, and Turkey. All other identified AvrStb6 isoforms were represented by one to eight isolates in the collection. Remarkably, 29 haplotypes and 25 AvrStb6 protein isoforms each were uniquely

present in single isolates in the collection, representing well over half of the identified AvrStb6 isoforms (Figure 1).

2.3 | Turkey is a hotspot of *AvrStb6* diversity

Fifty-seven *Z. tritici* isolates were sourced from smallholder farmers' wheat fields in Turkey as part of this study. From this sample, 29 *AvrStb6* haplotypes corresponding to 26 protein isoforms were identified. This

TABLE 1 Prevalence of AvrStb6 isoform I02 in the recent global *Zymoseptoria tritici* collection vs. in the earlier collections

Region of origin	Collection	Country/state of origin	Collection year(s)	Number of isolates	AvrStb6 I02 (%)
Western Europe	This study	England	2015–2017	135	94.8
		Scotland	2015–2017	14	92.9
		Ireland	2015–2017	8	87.5
		France	2015–2017	12	91.7
		Germany	2015–2017	12	66.7
	Brunner and McDonald (2018)	Switzerland	1999	29	51.7
	Zhong et al. (2017)	France	2009–2010	102	49
North America	This study	USA/Oregon	2016	48	89.6
	Brunner and McDonald (2018)	USA/Oregon	1990	56	0
Australia	This study	Tasmania	2014	58	96.6
	Brunner and McDonald (2018)	New South Wales	2001	27	74.1
South America	This study	Chile	2016	10	90
		Argentina	2016	10	20
		Uruguay	2016	10	30
Mediterranean	This study	Turkey	2013–2016	46	0

represents a far higher rate of gene and protein sequence diversity than found in any other region studied here, with more than half of all identified AvrStb6 isoforms being found in Turkey and 25 being unique to this region. The network diagram of *AvrStb6* haplotypes (Figure 2a) visualizes this high diversity, with both major branches largely consisting of haplotypes identified exclusively in the *Z. tritici* population from Turkey. Interestingly, isoform I02—the most prevalent isoform globally—was not observed in the Turkish population. This population therefore represents a notable break in the trend described above.

2.4 | *Stb6* resistance-breaking isoforms are widespread in the current population

We next aimed to determine whether the various identified AvrStb6 isoforms conferred avirulence or virulence towards wheat genotypes carrying the corresponding resistance gene *Stb6*. For this, for each of the nine AvrStb6 isoforms that were identified in more than one *Z. tritici* isolate in the collection (Figure S2) we randomly selected one isolate as representative for use in wheat infection bioassays. One exception was the most common isoform I02, for which two representative isolates, 2NIAB and R16.1 (collected in 2015 and 2016, respectively, from different commercial fields in the UK), were selected. The bioassays involved fungal inoculation of two pairs of wheat genotypes at the seedling stage, with the genotypes in each pair being genetically near identical but distinguished by the presence/absence of *Stb6*. One pair comprised wheat cv. Cadenza, carrying the resistance-conferring haplotype 1 of *Stb6* (Saintenac et al., 2018), and a mutant of cv. Cadenza that contains no functional *Stb6* due to a large CRISPR/Cas9-induced frameshift deletion in the first exon of this gene. The second pair comprised wheat landrace Chinese Spring (*Stb6*) and a near-isogenic line that contains the susceptibility haplotype 3 of *Stb6*

developed from a cross between Chinese Spring and a susceptible cv. Courtot. The previously characterized avirulent and virulent *Z. tritici* isolates IPO323 (Brading et al., 2002) and IPO88004 (Saintenac et al., 2018), possessing isoforms I01 and I44, respectively, were used as controls. Of the 10 isolates (representing nine different isoforms) tested, nine induced typical STB disease symptoms and fungal pycnidia on all tested wheat genotypes irrespectively of the presence/absence of *Stb6* (Figures 3 and S3). This included the isolate(s) representing I02, the most prevalent AvrStb6 isoform in our collection.

Other than the avirulent reference isolate IPO323 (I01), only one other isolate could potentially be defined as demonstrating reduced virulence on *Stb6* wheat, producing significantly fewer pycnidiospores on resistant wheat lines than those lacking *Stb6* (Figure 3a). This isolate possessed the AvrStb6 isoform I13, which shares the highest percentage amino acid identity with the known avirulence isoform I01 (Figure S2). One other isolate, possessing AvrStb6 isoform I03, which was classified as virulent, also induced lower levels of disease on the *Stb6*-containing wheat compared to their near-isogenic genotypes that lack *Stb6* as reflected by differences in the observed pycnidiospore counts (Figure 3). However, these differences were significant (at $p < 0.01$) only for the *Z. tritici* isolate T1-A2017.24 possessing the AvrStb6 isoform I13 and the control avirulent isolate IPO323.

2.5 | Avirulence isoforms of AvrStb6 are common on wheat genotypes irrespective of the *Stb6* resistance gene status

We wondered whether overrepresentation of AvrStb6 virulence isoforms in our isolates collection could have been due to most isolates potentially sampled from resistant, *Stb6*-containing wheat
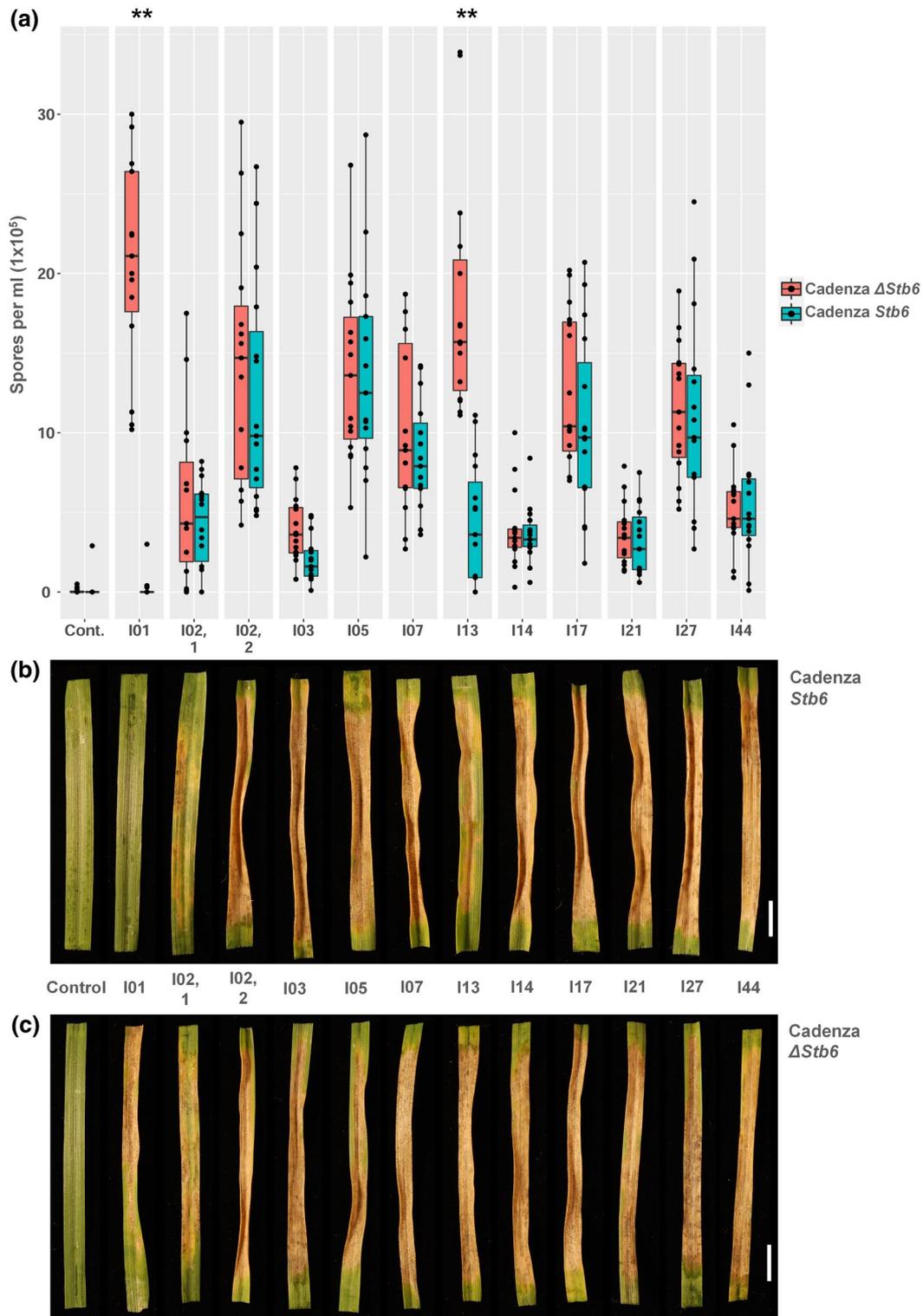
FIGURE 3 Wheat inoculation bioassay. Leaves of differential wheat genotypes, wildtype cv. Cadenza containing *Stb6* and a CRISPR/Cas9-induced deletion mutant (Cadenza Δ*Stb6*) that lacks the functional resistance gene in the same genetic background, were inoculated as young, 3-week-old seedlings with a selection of *Zymoseptoria tritici* isolates possessing different isoforms of AvrStb6. (a) Counts of pycnidiospores washed off the inoculated wheat leaves at 21 days postinoculation (dpi). Asterisks represent isolates with highly significant (**$p < 0.005$) differences in pycnidiospore counts between the resistant and susceptible genotypes. The most common isoform I02 was represented by the two isolates (labelled 1 and 2) originating from two different years and sampling locations. (b) and (c) Images of inoculated wheat leaves harvested at 21 dpi and incubated for 2 days under c.100% humidity to induce pycnidiation. Scale bar, 10 mm

cultivars. The cultivar information was available for 254 out of 381 sampled *Z. tritici* isolates, and it was possible to obtain 20 cultivars—sources of 210 isolates—for further study (Table S1). Ten of these cultivars have been characterized in the previous study (Saintenac et al., 2018) and their *Stb6* haplotype status and reaction to the avirulent strain IPO323 of *Z. tritici* are known (Table S1). We then resequenced *Stb6* following the procedure described in Saintenac et al. (2018) from the other 10 cultivars (Consort, Dickens, Gallant, Genesis, Kaseberg, KWS Cashel, Lumos, Marston, Solace, and Zulu) to identify their *Stb6* haplotype status (Table S1). It was determined that 9/20 of these cultivars possess disease resistance-conferring haplotype 1 of *Stb6* and they were sources of 73 (34.8%) *Z. tritici* isolates in our collection, whereas the other 11/20 analysed cultivars contained nonfunctional susceptibility haplotypes 3 or 7 of *Stb6* and they represented sources of 137 (65.2%) isolates (Table 2). Therefore, it appears that there was no overall bias in the wheat cultivars used in this study towards susceptible or resistant, and that a larger proportion of *Z. tritici* isolates was sampled from cultivars containing no functional *Stb6*.

TABLE 2 *Stb6* haplotype data for wheat cultivars used in this study

Wheat cultivar	Stb6 haplotype code[a]	Resistant (R)/ susceptible (S) haplotype	No. of isolates collected from the cultivar
Alchemy	7	S	1
Consort	7	S	17
Cordiale	7	S	13
Cougar	1	R	22
Crusoe	7	S	1
Dickens	1	R	22
Evolution	1	R	6
Gallant	7	S	7
Genesis	3	S	10
JB Diego	7	S	2
Kaseburg	7	S	52
KWS Cashel	3	S	21
KWS Santiago	1	R	4
KWS Siskin	1	R	13
Lumos	7	S	8
Marston	1	R	1
Reflection	7	S	5
Solace	1	R	1
Trapez	1	R	2
Zulu	1	R	2
Haplotype totals	1	R	73
	3	S	31
	7	S	106

[a]*Stb6* haplotypes are coded as in Saintenac et al. (2018).

2.6 | *AvrStb6* expression is highly variable between *Z. tritici* isolates

Our main hypothesis is that the breakdown of *Stb6*-mediated resistance by the current *Z. tritici* isolates analysed in this study (Figures 3 and S3) was due to the numerous changes in the AvrStb6 protein sequence identified (Figure 1). However, we also considered an alternative possibility whereby breakdown of *Stb6* resistance may have been due to the suppression of *AvrStb6* expression during infection. We therefore used a quantitative reverse transcription PCR (RT-qPCR) to compare the expression levels of *AvrStb6* in the 10 selected *Z. tritici* isolates (representing 10 different most frequent AvrStb6 isoforms) that were virulent on *Stb6* wheat versus an avirulent isolate IPO323 during infection of a fully susceptible wheat cv. Taichung 29 that does not possess *Stb6* (Ghaffary et al., 2012). As each of the 11 tested isolates has different infection dynamics on this wheat cultivar, the leaf tissues for use in RT-qPCR were sampled at different time points after fungal inoculation, depending on the isolate, when the first symptoms of the disease become visible. This infection phase was chosen as many *Z. tritici* effector genes, including *AvrStb6*, have previously been shown to display maximal expression during transition from the biotrophic to the necrotrophic phase (Rudd et al., 2015; Zhong et al., 2017).

RT-qPCR analysis revealed a high variability in *AvrStb6* expression between isolates (Figure S4). Expression levels for IPO323 (I01) were higher than for many other isolates, apart from the isolate representative of I21, although the statistical significance of these differences was not examined. Importantly, however, although the *AvrStb6* expression levels were highly variable between different isolates, the effector gene was expressed by all of them, although in WAI1822, carrying AvrStb6 isoform 14, expression was substantially suppressed (Figure S4). Overall, these findings argue against the mechanism of *Stb6* resistance breakdown being due to suppression of *AvrStb6* expression.

3 | DISCUSSION

Few studies have analysed the temporal changes in Avr factor prevalence driven by selection pressure from host species in pathogen populations. Often, these have a fairly narrow geographical area coverage. For example, studies into the changes in *Avr* gene prevalence in the fungal pathogen *Leptosphaeria maculans* of *Brassica* crops over time have focused on single countries such as Canada (Fernando et al., 2018) or Australia (Van de Wouw et al., 2018). On the other hand, studies with a global geographical scope often do not consider the impact of sampling time on changes in Avr factor prevalence. Here we analysed the diversity of the avirulence factor *AvrStb6* in the recent global *Z. tritici* populations. Moreover, we took advantage of the two similar published studies that analysed the population diversity of *AvrStb6* in earlier *Z. tritici* collections, sampled in 1990–2001 or 2009–2010 (Brunner & McDonald, 2018; Zhong et al., 2017). Comparing our data that was based on

the analysis of isolates sampled in 2013–2017 with that from these two previous studies revealed that large shifts in *AvrStb6* haplotype prevalence have taken place in multiple global regions over the relatively short time period between samplings. These include the avirulence isoform of AvrStb6 originally described from the reference isolate IPO323 (collected in the Netherlands in 1981) becoming potentially extinct in the recent *Z. tritici* populations, and emergence of multiple virulence isoforms with one becoming predominant globally. We hypothesize that these changes in *Z. tritici* populations may be imposed by a worldwide increase in the deployment of commercial wheat cultivars carrying *Stb6* (Chartrain et al., 2005; Saintenac et al., 2018), although comprehensive evidence for this hypothesis is currently unavailable.

The high proportion of AvrStb6 isoforms to haplotypes illustrates the high frequency of nonsynonymous mutations within the effector gene, which in turn suggests a strong selection pressure for the evolution of novel AvrStb6 protein isoforms that cannot be recognized by the corresponding wheat immune receptor Stb6. This phenomenon is well documented for other pathogen effectors, for instance with diversifying selection observed for ToxA, Tox1, and Tox3 of *Parastagonospora nodorum* (McDonald et al., 2013; Stukenbrock & McDonald, 2007), NIP1 of *Rhynchosporium commune* (Schürch et al., 2004), and AvrP123 and AvrP4 of the flax rust pathogen *Melampsora lini* (Barrett et al., 2009). In several fungal pathogens, RIPs have been shown to be responsible for the evolution of virulence due to the diversification of effector gene sequences, including the introduction of multiple premature stop codons (Fudal et al., 2009). Given the transposon-rich, highly dynamic region of the genome in which *AvrStb6* is located (Sánchez-Vallet et al., 2018), it seems possible that a similar mechanism has contributed to the diversification of this *Z. tritici* effector. However, an in silico analysis suggests a low frequency of RIPs in the *AvrStb6* genomic region (Figure S5). Moreover, we found that the secretion signal peptide region and all the cysteine residues in AvrStb6 were highly conserved (Figure 1), whilst premature stop codon mutations within the gene were extremely rare. The signal peptide of effectors is required for their secretion into the host apoplast, whilst the formation of disulphide bonds between cysteines is thought to help to stabilize the protein in the harsh alkaline pH apoplastic environment. In addition, using RT-qPCR analysis we showed that *AvrStb6* was expressed in a set of *Z. tritici* isolates representing the most frequently occurring isoforms of this effector during a phase transition from biotrophy to necrotrophy (Figure S4). Whilst efforts to demonstrate a role for AvrStb6 isoforms in virulence have to date proved unsuccessful (Kema et al., 2018), these findings combined suggest an evolutionary pressure to maintain a functional effector, implying the potential contribution of AvrStb6 to fungal virulence or fitness, perhaps under certain, as yet unknown, environmental conditions.

Our study revealed the prevalence of a single AvrStb6 isoform, I02, in the global population of *Z. tritici*. This isoform was found to be most common in all countries of western Europe from which the isolates were sourced, as well as Australia, the United States, and Chile. It is unknown why this isoform specifically became so prevalent in

multiple regions around the world recently, given the ability of isolates carrying other isoforms to also evade *Stb6*-mediated resistance (Figure 3). However, the limited diversity of *Stb6* haplotypes (*n* = 3) in the host wheat plants sampled (Table 2) may suggest that AvrStb6 isoform I02 is specifically adapted for successful infection of wheat possessing these *Stb6* haplotypes. The virulence isoform I02 may also be the best-adapted for carrying out its as-yet unknown effector function. Notably, sequencing of *AvrStb6* from *Z. tritici* isolates sourced from Oregon, United States, in 1990 failed to identify a single occurrence of I02 (Table 1). This is in sharp contrast to our study, which identified prevalence of this isoform, found in 82.7% of the current isolates in the Oregon part of our collection. This population shift, combined with the fact that in all cases I02 was coded by a single haplotype H02, may suggest the introduction and rapid proliferation of this virulence *AvrStb6* haplotype throughout the population of *Z. tritici* in Oregon, as occurred, for example, with the recent accidental importation of *Pyricularia oryzae*, a wheat blast pathogen, from Brazil to Bangladesh (Islam et al., 2016).

Our data also revealed a profound increase in the prevalence of AvrStb6 isoform I02 in the global recent (2013–2017) *Z. tritici* population compared to that in the older collections comprising isolates sampled in 1990–2001 (Brunner & McDonald, 2018) or 2009–2010 (Zhong et al., 2017) (Table 1). One exception to this is Australia, where a prevalence of AvrStb6 I02 in the local *Z. tritici* population sampled in 2001 was already evident (Zhan et al., 2005) (Table 1). Interestingly, in addition to the increasing prevalence of a single isoform I02, the overall diversity of AvrStb6 isoforms seem to have decreased over time. Thus, for example, previous studies of not current isolates by Brunner and McDonald (2018) and Zhong et al. (2017) identified 30 and 18 AvrStb6 isoforms in the populations comprising 142 or 103 isolates, respectively. These numbers are substantially higher than the 42 isoforms identified from 381 current isolates analysed in this study. Such a large shift in the *AvrStb6* population diversity that occurred over a relatively short period of time and the increased fixation of AvrStb6 I02 in the population could have been caused by the decreased genetic diversity of wheat due to breeding (Fu & Somers, 2009) and widespread global deployment of *Stb6*-containing cultivars by arable farmers (Chartrain et al., 2005; Saintenac et al., 2018). Other studies have shown changes in the Avr factor prevalence, such as an increased incidence of *AvrLm4-7* in *L. maculans* from 47.2% in 2005–2006 (Dilmaghani et al., 2009) to 89.7% in 2010–2011 (Liban et al., 2016) in western Canada, demonstrating the ability of pathogens to adapt rapidly to changing conditions in host populations. However, to our knowledge this is the first time that a shift of such significance has been shown to occur across a global population of a plant pathogen.

The analysis of *AvrStb6* haplotypes did not identify I02 as the dominant isoform in Argentina and Uruguay, and this isoform was completely absent from the Turkish *Z. tritici* population. It is possible that the local wheat cultivars used for sampling *Z. tritici* isolates for this study in these countries may have unique genetic makeups, although it was not possible to test this hypothesis in this study. The

Turkish population is of particular interest, given it has a substantially higher *AvrStb6* haplotype diversity compared to those in the other wheat-growing regions studied here. Whilst the exact reasons for this difference are unknown, we hypothesize that the widespread use of local genetically diverse landraces, instead of genetically more similar commercial wheat cultivars, in Turkey is a driver for matching diversification of *Z. tritici* genomes. The location of Turkey near or at the hypothesized geographic region of the origin of wheat (Shewry, 2009) and *Z. tritici* (Stukenbrock et al., 2006) may also be a cause for the high diversity of *AvrStb6* haplotypes observed here because origins of species often also correspond to the centres of their genetic diversity.

A recent study (Kema et al., 2018) suggested that the *Z. tritici* pathogenicity towards wheat cultivars carrying the disease resistance gene *Stb6* was associated with changes at the amino acid positions 41 and 43 in the AvrStb6 protein. The new data obtained here support these findings and we confirm that isolates possessing isoforms I01 and I13 having G and D at positions 41 and 43, respectively (Figure S2), were either avirulent or generated significantly fewer pycnidiospores on wheat cultivars possessing *Stb6* (Figure 3). However, we also found that the *Z. tritici* isolates expressing AvrStb6 isoform I03, I17, or I44, having E or V instead of G at position 41 but D at position 43, were fully virulent on *Stb6* wheat (Figure S2). This suggests that amino acid changes in AvrStb6 at position 41 alone may be responsible for the *Stb6* resistance-breaking ability of *Z. tritici* isolates. It would be interesting to test this hypothesis through targeted mutagenesis in follow-on studies, or indeed through testing of those Turkish isolates also possessing AvrStb6 isoforms with G and D at positions 41 and 43 (Figure 1).

The number of *Z. tritici* isolates analysed in this study and the failure to identify AvrStb6 isoform I01 in the collection suggest that this isoform has been eliminated from the modern *Z. tritici* population worldwide. However, the *AvrStb6* haplotype network analysis indicates that the true diversity of *AvrStb6* may be higher than identified as in some cases multiple mutations were found to separate the neighbouring haplotypes in the network (Figure 2a). A significant amount of undiscovered sequence variation in *AvrStb6* may still be present in the global *Z. tritici* population. However, one of the main conclusions from our study is that the wheat disease resistance gene *Stb6* has currently been almost completely overcome due to the predominance of fungal isolates carrying resistance-breaking AvrStb6 isoforms in the modern *Z. tritici* populations around the world. This suggests that, despite the ability of *Z. tritici* to sexually reproduce on resistant hosts (Kema et al., 2018), Avr factors tend to be eliminated in subsequent populations.

4 | EXPERIMENTAL PROCEDURES

4.1 | *Z. tritici* isolates collection

The 381 *Z. tritici* isolates used in this study were collected from naturally infected wheat fields in the UK (*n* = 150), Ireland (*n* = 8), France (*n* = 12), Germany (*n* = 12), Australia (*n* = 58), and Turkey (*n* = 57), and from single fields in Chile (*n* = 10), Argentina (*n* = 10), Uruguay (*n* = 10), and the United States (*n* = 52). Most of these isolates were collected between 2015 and 2017, with the exception of isolates collected in Australia (2014) and Turkey (2013–2016), and the control isolates IPO323 (avirulent on *Stb6* wheat) and IPO88004 (virulent on *Stb6* wheat) collected in 1981 and 1988 in the Netherlands and Ethiopia, respectively (Goodwin et al., 2011; Kema & van Silfhout, 1997). Stocks of *Z. tritici* isolates were stored as blastospore water suspensions in 50% (vol/vol) glycerol at −80 °C.

4.2 | Sequence, diversity, and phylogenetic analysis of *AvrStb6*

Genomic DNA extraction was carried out from *Z. tritici* isolates as previously described (Rudd et al., 2010). Primers for PCR amplification and sequencing of the *AvrStb6* gene were designed in the upstream and downstream untranslated regions (UTRs) of the gene using publicly available and own whole-genome sequencing data for European *Z. tritici* isolates (Chen et al., 2019, Rothamsted Research, personal communication). Primers avrstb6.f1 and avrstb6.r1 were selected for amplification of *AvrStb6* from European isolates, and primers avrstb6.f3 and avrstb6.r1 were used to amplify *AvrStb6* from North and South American isolates (Table S2). All amplifications were done using Phusion High-Fidelity DNA polymerase (New England Biolabs UK Ltd). PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) and Sanger sequenced in house or at Eurofins Genomics UK Ltd. *AvrStb6* sequences from the Australian *Z. tritici* isolates were extracted from the whole-genome sequencing data (NCBI BioProject accession number PRJNA480739; McDonald et al., 2019).

AvrStb6 haplotype and the corresponding protein isoform sequences were aligned using MAFFT v. 7.388 (Katoh & Standley, 2013) with the phylogenetic tree assembled using PhyML (Guindon & Gascuel, 2003). The *AvrStb6* sequence from the *Z. tritici* isolate IPO323 was used as a reference for MAFFT alignments, whilst a paralogue of *AvrStb6* present on chromosome 10 (gene *Mycgr3G82331*; Brunner & McDonald, 2018) was used to root the phylogenetic tree. A TCS haplotype network (Clement et al., 2000) was created to visualize the diversity of *AvrStb6* haplotypes identified in the population using PopArt v. 1.7 (Leigh & Bryant, 2015). Coding regions were annotated and translated to amino acid sequence using Geneious v. 10.2.3 (Biomatters Ltd). RIP analysis of the region of *Z. tritici* chromosome 5 containing *AvrStb6* was carried out using RIPper software (van Wyk et al., 2019).

The *AvrStb6* sequence diversity data obtained here was compared to the similar previously published data sets for the historic *Z. tritici* collections. The *AvrStb6* haplotype sequence obtained in Zhong et al. (2017) was kindly provided by Daniel Croll (University of Neuchâtel, Switzerland) and those obtained in Brunner and McDonald (2018) were downloaded from the NCBI PopSet Database (accession number 1,337,388,362).

4.3 | Generation of wheat genetic material

A wheat Chinese Spring near-isogenic line that carries the suscep-tibility haplotype 3 at the *Stb6* locus was obtained following five backcrosses starting with F_1 Chinese Spring × Courtot. In each gen-eration, plants were assessed for their responses to the avirulent *Z. tritici* isolate IPO323 and genotyped with simple sequence repeat marker GWM369 to maintain the susceptibility haplotype of *Stb6* in the progenies. A single BC_5F_1 plant heterozygous at the *Stb6* locus was self-pollinated. BC_5F_2 plants homozygous for the susceptibility haplotype of *Stb6* were selfed and constitute the near-isogenic line used in this study.

The Cadenza Δ*Stb6* mutant was produced by targeting the first exon of the *Stb6* gene with CRISPR/Cas9 at five sites (Figure S6). The sgRNA constructs, carrying two sgRNAs each (annotated sequences are available on figshare), were assembled using the pENTR4-sgRNA4 vector as previously described (Zhou et al., 2014). All three sgRNA plasmids were co-delivered along with pCas9-GFP (Zhang et al., 2019), encoding the wheat codon-optimized Cas9 fused in frame at the C-terminus with green fluorescent protein (GFP), and pRRes1.111 (Alotaibi et al., 2018), which directs expression of the *bar* selectable marker gene into immature wheat embryos of wheat cv. Cadenza using biolistics essentially as described in Sparks and Doherty (2020). CRISPR/Cas9 mutagenized T_0 lines were genotyped for indels using the PCR band-shift assay (Nekrasov et al., 2017). Cas9, sgRNA, and *bar* transgenes were segregated out in subsequent generations and the homozygous Cadenza Δ*Stb6* T_2 line was used for phenotyping with *Z. tritici*.

4.4 | Fungal bioassays

Leaves of 3-week-old wheat seedlings were inoculated with *Z. tritici* blastospore suspensions at 10^7 spores/ml as previously described (Keon et al., 2007). Wheat cv. Cadenza and the landrace Chinese Spring, both carrying *Stb6*, were used as resistant controls. A CRISPR/Cas9-induced Cadenza Δ*Stb6* mutant and a near-isogenic line in the Chinese Spring background that carries a susceptibility haplotype at the *Stb6* locus were used as susceptible controls. A split-plot randomized experimental design was used when carrying out the bioassays. Analysis using both sets of cultivars was carried out with five biological replicates per *Z. tritici* isolate tested. For cv. Cadenza lines, three independent repeats of this bioassay were car-ried out, whilst a single repeat was carried out using the Chinese Spring near-isogenic lines.

STB disease symptoms on each inoculated leaf were visu-ally assessed at 21 days postinoculation (dpi) as previously de-scribed (Lee et al., 2015). To complement the visual disease assessment, we carried out pycnidiospore wash counts as follows. Approximately 6-cm long segments were cut from the inoculated leaves and these were exposed to high relative humidity at 15 °C for 48 hr in the dark to induce pycnidiation. Pycnidiospores were washed off the infected leaves by addition of 2 ml of distilled water supplemented with 0.01% Tween 20 followed by vortex-ing for 30 s. Optical density at 600 nm (OD_{600}) of the harvested pycnidiospore suspensions was measured by spectrophotometer. This was converted to spores/ml by comparing against a stan-dard curve produced by measuring OD_{600} of control suspensions containing defined pycnidiospore numbers as counted using a haemocytometer.

4.5 | Wheat *Stb6* haplotype analysis

Stb6 haplotype data for 10 wheat cultivars, sources of the *Z. tritici* isolates used here, were available from the recently published study (Saintenac et al., 2018). For 10 other cultivars, with previously un-known *Stb6* status, we extracted genomic DNA from 3-week-old wheat plants and resequenced *Stb6* exons following previously pub-lished methodology (Saintenac et al., 2018). Wheat cultivar infor-mation for *Z. tritici* isolates from Australia, Turkey, and a few from western Europe was not available.

4.6 | Analysis of *AvrStb6* expression during the infection of wheat

Three-week-old seedlings of wheat cv. Taichung 29, which is highly susceptible to *Z. tritici* and contains no known genes/quan-titative trait loci for resistance to STB (Ghaffary et al., 2012), were inoculated with *Z. tritici* spore suspension at 10^7 spores/ml as previously described (Keon et al., 2007). At the emergence of visible symptoms on the inoculated leaves between 12 and 16 dpi (depending on the particular isolate), the leaves were harvested and snap-frozen in liquid nitrogen. Total RNA was extracted using TRIzol (Fisher Scientific) following the manufacturer's instruc-tions, and any potential traces of genomic DNA were removed using a TURBO DNA-free kit (Fisher Scientific). First-strand cDNA was synthesized from 1 μg of total RNA using SuperScript IV re-verse transcriptase (Fisher Scientific). First-strand cDNA prepara-tions were diluted 1:10 with RNase-free water and subjected to qPCR using SYBR Green JumpStart Taq ReadyMix (Merck Life Science UK Ltd), with primers specified in Table S2. Three bio-logical and three technical replicates were carried out for each sample, with relative expression of *AvrStb6* to the *Z. tritici* house-keeping gene *glucose-6-phosphate 1-dehydrogenase* (*G6PDH*; gene ID Mycgr3G100879) calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

DATA AVAILABILITY STATEMENT

The sequencing data that support the findings will be openly available in GenBank at https://www.ncbi.nlm.nih.gov/nucleotide/ following an embargo from the date of publication, accession numbers: MT856831–MT856877. Annotated sgRNA construct sequences in the GenBank format are available on figshare at https://doi.org/10.6084/m9.figshare.12964589.v1.

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REFERENCES

Alotaibi, S.S., Sparks, C.A., Parry, M.A.J., Simkin, A.J. & Raines, C.A. (2018) Identification of leaf promoters for use in transgenic wheat. *Plants*, 7, 27.

Barrett, L.G., Thrall, P.H., Dodds, P.N., van der Merwe, M., Linde, C.C., Lawrence, G.J. et al. (2009) Diversity and evolution of effector loci in natural populations of the plant pathogen *Melampsora lini*. *Molecular Biology and Evolution*, 26, 2499–2513.

Blondeau, K., Blaise, F., Graille, M., Kale, S.D., Linglin, J., Ollivier, B. et al. (2015) Crystal structure of the effector AvrLm4-7 of *Leptosphaeria maculans* reveals insights into its translocation into plant cells and recognition by resistance proteins. *The Plant Journal*, 83, 610–624.

Brading, P.A., Verstappen, E.C.P., Kema, G.H.J. & Brown, J.K.M. (2002) A gene-for-gene relationship between wheat and *Mycosphaerella graminicola*, the Septoria tritici blotch pathogen. *Phytopathology*, 92, 439–445.

Brunner, P.C. & McDonald, B.A. (2018) Evolutionary analyses of the avirulence effector AvrStb6 in global populations of *Zymoseptoria tritici* identify candidate amino acids involved in recognition. *Molecular Plant Pathology*, 19, 1836–1846.

Chartrain, L., Brading, P.A. & Brown, J.K.M. (2005) Presence of the *Stb6* gene for resistance to septoria tritici blotch (*Mycosphaerella graminicola*) in cultivars used in wheat-breeding programmes worldwide. *Plant Pathology*, 54, 134–143.

Clement, M., Posada, D. & Crandall, K.A. (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, 9, 1657–1659.

Cook, D.E., Mesarich, C.H. & Thomma, B.P.H.J. (2015) Understanding plant immunity as a surveillance system to detect invasion. *Annual Review of Phytopathology*, 53, 541–563.

Cowger, C., Hoffer, M.E. & Mundt, C.C. (2000) Specific adaptation by *Mycosphaerella graminicola* to a resistant wheat cultivar. *Plant Pathology*, 49, 445–451.

Dilmaghani, A., Balesdent, M.H., Didier, J.P., Wu, C., Davey, J., Barbetti, M.J. et al. (2009) The *Leptosphaeria maculans–Leptosphaeria biglobosa* species complex in the American continent. *Plant Pathology*, 58, 1044–1058.

Djamei, A., Schipper, K., Rabe, F., Ghosh, A., Vincon, V., Kahnt, J. et al. (2011) Metabolic priming by a secreted fungal effector. *Nature*, 478, 395–398.

Dong, S., Raffaele, S. & Kamoun, S. (2015) The two-speed genomes of filamentous pathogens: waltz with plants. *Current Opinion in Genetics & Development*, 35, 57–65.

Fernando, W.G.D., Zhang, X., Selin, C., Zou, Z., Liban, S.H., McLaren, D.L. et al. (2018) A six-year investigation of the dynamics of avirulence allele profiles, blackleg incidence, and mating type alleles of *Leptosphaeria maculans* populations associated with canola crops in Manitoba, Canada. *Plant Disease*, 102, 790–798.

Fu, Y.-B. & Somers, D.J. (2009) Genome-wide reduction of genetic diversity in wheat breeding. *Crop Science*, 49, 161–168.

Fudal, I., Ross, S., Brun, H., Besnard, A.-L., Ermel, M., Kuhn, M.-L. et al. (2009) Repeat-induced point mutation (RIP) as an alternative mechanism of evolution toward virulence in *Leptosphaeria maculans*. *Molecular Plant-Microbe Interactions*, 22, 932–941.

Ghaffary, S.M.T., Faris, J.D., Friesen, T.L., Visser, R.G.F., van der Lee, T.A.J., Robert, O. et al. (2012) New broad-spectrum resistance to Septoria tritici blotch derived from synthetic hexaploid wheat. *Theoretical and Applied Genetics*, 124, 125–142.

Goodwin, S.B., Ben M'Barek, S., Dhillon, B., Wittenberg, A.H.J., Crane, C.F., Hane, J.K. et al. (2011) Finished genome of the fungal wheat pathogen *Mycosphaerella graminicola* reveals dispensome structure, chromosome plasticity, and stealth pathogenesis. *PLoS Genetics*, 7, e1002070.

Guindon, S. & Gascuel, O. (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology*, 52, 696–704.

Hovmøller, M.S. & Justesen, A.F. (2007) Rates of evolution of avirulence phenotypes and DNA markers in a northwest European population of *Puccinia striiformis* f. sp. *tritici*. *Molecular Ecology*, 16, 4637–4647.

Islam, M.T., Croll, D., Gladieux, P., Soanes, D.M., Persoons, A., Bhattacharjee, P. et al. (2016) Emergence of wheat blast in Bangladesh was caused by a South American lineage of *Magnaporthe oryzae*. *BMC Biology*, 14, 84.

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

Kanyuka, K. & Rudd, J.J. (2019) Cell surface immune receptors: the guardians of the plant's extracellular spaces. *Current Opinion in Plant Biology*, 50, 1–8.

Katoh, K. & Standley, D.M. (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution*, 30, 772–780.

Kema, G.H.J., Mirzadi Gohari, A., Aouini, L., Gibriel, H.A., Ware, S.B., van Den Bosch, F. et al. (2018) Stress and sexual reproduction affect the dynamics of the wheat pathogen effector AvrStb6 and strobilurin resistance. *Nature Genetics*, 50, 375–380.

Kema, G.H.J. & van Silfhout, C.H. (1997) Genetic variation for virulence and resistance in the wheat–*Mycosphaerella graminicola* pathosystem III. Comparative seedling and adult plant experiments. *Phytopathology*, 87, 266–272.

Keon, J., Antoniw, J., Carzaniga, R., Deller, S., Ward, J.L., Baker, J.M. et al. (2007) Transcriptional adaptation of *Mycosphaerella graminicola* to programmed cell death (PCD) of its susceptible wheat host. *Molecular Plant-Microbe Interactions*, 20, 178–193.

Kleemann, J., Rincon-Rivera, L.J., Takahara, H., Neumann, U., van Themaat, E.V.L., van der Does, H.C. et al. (2012) Sequential delivery of host-induced virulence effectors by appressoria and intracellular hyphae of the phytopathogen *Colletotrichum higginsianum*. *PLoS Pathogens*, 8, e1002643.

Lee, W.-S., Rudd, J.J. & Kanyuka, K. (2015) Virus induced gene silencing (VIGS) for functional analysis of wheat genes involved in *Zymoseptoria tritici* susceptibility and resistance. *Fungal Genetics and Biology*, 79, 84–88.

Leigh, J.W. & Bryant, D. (2015) POPART: full-feature software for haplotype network construction. *Methods in Ecology and Evolution*, 6, 1110–1116.

Liban, S.H., Cross, D.J., Kutcher, H.R., Peng, G. & Fernando, W.G.D. (2016) Race structure and frequency of avirulence genes in the western Canadian *Leptosphaeria maculans* pathogen population, the causal agent of blackleg in brassica species. *Plant Pathology*, 65, 1161–1169.

Livak, K.J. & Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, 25, 402–408.

Luderer, R., Takken, F.L.W., de Wit, P.J.G.M. & Joosten, M.H.A.J. (2002) *Cladosporium fulvum* overcomes *Cf-2*-mediated resistance by producing truncated AVR2 elicitor proteins. *Molecular Microbiology*, 45, 875–884.

Marshall, R., Kombrink, A., Motteram, J., Loza-Reyes, E., Lucas, J., Hammond-Kosack, K.E. et al. (2011) Analysis of two in planta expressed LysM effector homologs from the fungus *Mycosphaerella graminicola* reveals novel functional properties and varying contributions to virulence on wheat. *Plant Physiology*, 156, 756–769.

McDonald, M.C., Oliver, R.P., Friesen, T.L., Brunner, P.C. & McDonald, B.A. (2013) Global diversity and distribution of three necrotrophic effectors in *Phaeosphaeria nodorum* and related species. *New Phytologist*, 199, 241–251.

McDonald, M.C., Renkin, M., Spackman, M., Orchard, B., Croll, D., Solomon, P.S. et al. (2019) Rapid parallel evolution of azole fungicide resistance in Australian populations of the wheat pathogen *Zymoseptoria tritici*. *Applied and Environmental Microbiology*, 85, e01908-18.

Nekrasov, V., Wang, C., Win, J., Lanz, C., Weigel, D. & Kamoun, S. (2017) Rapid generation of a transgene-free powdery mildew resistant tomato by genome deletion. *Scientific Reports*, 7, 482.

Plissonneau, C., Blaise, F., Ollivier, B., Leflon, M., Carpezat, J., Rouxel, T. et al. (2017) Unusual evolutionary mechanisms to escape effector-triggered immunity in the fungal phytopathogen *Leptosphaeria maculans*. *Molecular Ecology*, 26, 2183–2198.

Presti, L.L., Lanver, D., Schweizer, G., Tanaka, S., Liang, L., Tollot, M. et al. (2015) Fungal effectors and plant susceptibility. *Annual Review of Plant Biology*, 66, 513–545.

Qutob, D., Patrick Chapman, B. & Gijzen, M. (2013) Transgenerational gene silencing causes gain of virulence in a plant pathogen. *Nature Communications*, 4, 1349.

Rouxel, T., Grandaubert, J., Hane, J.K., Hoede, C., Van de Wouw, A.P., Couloux, A. et al. (2011) Effector diversification within compartments of the *Leptosphaeria maculans* genome affected by repeat-induced point mutations. *Nature Communications*, 2, 202.

Rudd, J.J., Antoniw, J., Marshall, R., Motteram, J., Fraaije, B. & Hammond-Kosack, K. (2010) Identification and characterisation of *Mycosphaerella graminicola* secreted or surface-associated proteins with variable intragenic coding repeats. *Fungal Genetics and Biology*, 47, 19–32.

Rudd, J.J., Kanyuka, K., Hassani-Pak, K., Derbyshire, M., Andongabo, A., Devonshire, J. et al. (2015) Transcriptome and metabolite profiling of the infection cycle of *Zymoseptoria tritici* on wheat reveals a biphasic interaction with plant immunity involving differential pathogen chromosomal contributions and a variation on the hemibiotrophic lifestyle definition. *Plant Physiology*, 167, 1158–1185.

Saintenac, C., Lee, W.-S., Cambon, F., Rudd, J.J., King, R.C., Marande, W. et al. (2018) Wheat receptor-kinase-like protein Stb6 controls gene-for-gene resistance to fungal pathogen *Zymoseptoria tritici*. *Nature Genetics*, 50, 368–374.

Sánchez-Vallet, A., Fouché, S., Fudal, I., Hartmann, F.E., Soyer, J.L., Tellier, A. et al. (2018) The genome biology of effector gene evolution in filamentous plant pathogens. *Annual Review of Phytopathology*, 56, 21–40.

Schürch, S., Linde, C.C., Knogge, W., Jackson, L.F. & McDonald, B.A. (2004) Molecular population genetic analysis differentiates two virulence mechanisms of the fungal avirulence gene *NIP1*. *Molecular Plant-Microbe Interactions*, 17, 1114–1125.

Shewry, P.R. (2009) Concomitant increases of the developing seed phosphoenolpyruvate carboxylase activity and the seed protein content of field-grown wheat with nitrogen supply. *Wheat Journal of Experimental Botany*, 60, 1537–1553.

Sparks, C.A. & Doherty, A. (2020) Genetic transformation of common wheat (*Triticum aestivum* L.) using biolistics. *Methods in Molecular Biology*, 2124, 229–250.

Stukenbrock, E.H., Banke, S., Javan-Nikkhah, M. & McDonald, B.A. (2006) Origin and domestication of the fungal wheat pathogen *Mycosphaerella graminicola* via sympatric speciation. *Molecular Biology and Evolution*, 24, 398–411.

Stukenbrock, E.H. & McDonald, B.A. (2007) Geographical variation and positive diversifying selection in the host-specific toxin SnToxA. *Molecular Plant Pathology*, 8, 321–332.

Torriani, S.F.F., Melichar, J.P.E., Mills, C., Pain, N., Sierotzki, H. & Courbot, M. (2015) *Zymoseptoria tritici*: a major threat to wheat production, integrated approaches to control. *Fungal Genetics and Biology*, 79, 8–12.

Van de Wouw, A.P., Howlett, B.J. & Idnurm, A. (2018) Changes in allele frequencies of avirulence genes in the blackleg fungus, *Leptosphaeria maculans*, over two decades in Australia. *Crop and Pasture Science*, 69, 20.

Wang, L., Chen, H., Li, J., Shu, H., Zhang, X., Wang, Y. et al. (2020) Effector gene silencing mediated by histone methylation underpins host adaptation in an oomycete plant pathogen. *Nucleic Acids Research*, 48, 1790–1799.

van Wyk, S., Harrison, C.H., Wingfield, B.D., De Vos, L., van der Merwe, N.A. & Steenkamp, E.T. (2019) The RIPper, a web-based tool for genome-wide quantification of Repeat-Induced Point (RIP) mutations. *PeerJ*, 7, e7447.

Zhan, J., Linde, C.C., Jürgens, T., Merz, U., Steinebrunner, F. & McDonald, B.A. (2005) Variation for neutral markers is correlated with variation for quantitative traits in the plant pathogenic fungus *Mycosphaerella graminicola*. *Molecular Ecology*, 14, 2683–2693.

Zhang, S., Wang, L., Wu, W., He, L., Yang, X. & Pan, Q. (2015) Function and evolution of *Magnaporthe oryzae* avirulence gene *AvrPib* responding to the rice blast resistance gene *Pib*. *Scientific Reports*, 5, 11642.

Zhang, Z., Hua, L., Gupta, A., Tricoli, D., Edwards, K.J., Yang, B. et al. (2019) Development of an *Agrobacterium*-delivered CRISPR/Cas9 system for wheat genome editing. *Plant Biotechnology Journal*, 17, 1623–1635.

Zhong, Z., Marcel, T.C., Hartmann, F.E., Ma, X., Plissonneau, C., Zala, M. et al. (2017) A small secreted protein in *Zymoseptoria tritici* is responsible for avirulence on wheat cultivars carrying the *Stb6* resistance gene. *New Phytologist*, 214, 619–631.

Zhou, H., Liu, B., Weeks, D.P., Spalding, M.H. & Yang, B. (2014) Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. *Nucleic Acids Research*, 42, 10903–10914.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.