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Kema, G. H. J., Mirzadi Gohari, A., Aouini, L., Gibriel, H. A. Y., Ware, S. B., Van Den Bosch, F., Manning-Smith, R., Alonso Chavez, V., Helps, J., Ben M'Barek, S., Mehrabi, R., Diaz-Trujillo, C., Zamani, E., Schouten, H. J., Van Der Lee, T. A. J., Waalwijk, C., de Waard, M. A., de Wit, P. J. G. M., Verstappen, E. C. P., Thomma, B. P. H. J., Meijer, H. J. G. and Seidl, M.I F. 2018. Stress and sexual reproduction affect the dynamics of the wheat pathogen effector AvrStb6 and strobilurin resistance. *Nature Genetics.* 50 (3), pp. 375-380.

The publisher's version can be accessed at:

- https://dx.doi.org/10.1038/s41588-018-0052-9

The output can be accessed at: https://repository.rothamsted.ac.uk/item/8w277/stress-and-sexual-reproduction-affect-the-dynamics-of-the-wheat-pathogen-effector-avrstb6-and-strobilurin-resistance.

Stress and sexual reproduction affect the dynamics of the wheat pathogen effector AvrStb6 and strobilurin resistance

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Host resistance and fungicide treatments are cornerstones of plant-disease control. Here, we show that these treatments allow sex and modulate parenthood in the fungal wheat pathogen *Zymoseptoria tritici*. We demonstrate that the *Z. tritici*–wheat interaction complies with the gene-for-gene model by identifying the effector AvrStb6, which is recognized by the wheat resistance protein Stb6. Recognition triggers host resistance, thus implying removal of avirulent strains from pathogen populations. However, *Z. tritici* crosses on wheat show that sex occurs even with an avirulent parent, and avirulence alleles are thereby retained in subsequent populations. Crossing fungicide-sensitive and fungicide-resistant isolates under fungicide pressure results in a rapid increase in resistance-allele frequency. Isolates under selection always act as male donors, and thus disease control modulates parenthood. Modeling these observations for agricultural and natural environments reveals extended durability of host resistance and rapid emergence of fungicide resistance. Therefore, fungal sex has major implications for disease control.

Sexual reproduction is common in nearly all branches of the eukaryotic tree of life, including microbial organisms such as fungi[1,2], and is considered an important driver of rapid adaption to novel or changing environments[3]. Dothideomycete fungi are the largest and most ecologically diverse group of ascomycetes, comprising approximately 20,000 species[4], most of which reproduce sexually and asexually. One notable member is the plant pathogen *Zymoseptoria tritici*, which causes septoria tritici blotch in wheat. At the onset of the wheat growing season, *Z. tritici* produces airborne sexual ascospores, which form genetically diverse founding populations in commercial wheat fields[5–7], and splash-dispersed asexual conidia, which drive epidemics during the growing season[8]. Fungicides and host resistance are paramount for disease control. To date, 21 resistance genes to septoria tritici blotch (denoted *Stb* genes) have been identified (Supplementary Table 1) and mapped, and *Stb6*, which is ubiquitous in European wheat cultivars[9], is the first cloned resistance gene[10].

However, the molecular processes underlying the *Z. tritici*–wheat interaction are still relatively poorly understood[4,11–13].

Gene-for-gene (GFG) interaction models have been suggested for a plethora of plant–pathogen interactions[14], but genetic verification has been provided for only a limited number of pathosystems[15,16]. After more than a decade of genetic studies[4,6,12,17–20], we report the map-based cloning of what is, to our knowledge, the first described *Z. tritici* avirulence effector, AvrStb6, which triggers Stb6-mediated immunity[10] underlying GFG in the *Z. tritici*–wheat interaction. We have previously developed a mapping population between *Z. tritici* isolates IPO323 and IPO94269 (refs [4,12,17,19]), which we saturated here with Diversity Array Technology (DArTseq) markers (Supplementary Tables 2–4 and Supplementary Fig. 1) and mapped a putative avirulence effector gene on the tip of chromosome 5 (Table 1, Fig. 1 and Supplementary Note). Public RNA-seq data[21] were used to predict a single gene candidate (four exons; Supplementary Note and Supplementary Figs. 2–4), which was highly expressed in planta, encoding a small secreted protein (82 amino acids (aa), 12 cysteines, mature size 63 aa; Fig. 1). Deletion of *AvrStb6* in the avirulent strain IPO323 resulted in compatibility on cultivar (cv.) Shafir, which carries *Stb6*, thus identifying *AvrStb6* as the candidate avirulence effector. Introducing *AvrStb6* into the compatible strain IPO94269 resulted in incompatibility on cv. Shafir, thereby demonstrating that AvrStb6 is recognized by Stb6 (Fig. 1 and Supplementary Fig. 5). Recently, *AvrStb6* has also been identified through a genome-wide association study and subsequent ectopic integration in a virulent *Z. tritici* strain[22]. Analyses of the IPO323/IPO94269 mapping population and a panel of *Z. tritici* isolates suggested that the pathogenicity on cultivars carrying *Stb6* was consistent with two amino acid changes in the AvrStb6 protein (Supplementary Table 5 and Supplementary Fig. 6).

To generate segregating populations, *Z. tritici* isolates were crossed in planta (additional information in Supplementary Note), similarly to sex in nature[4–7,12,15,17–19]. Apart from demonstrating a GFG interaction between wheat and *Z. tritici*, we observed

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Table 1 | Inheritance of markers in *Z. tritici* mapping populations

Marker	IPO323	IPO94269	Progeny on bread wheat cv. Obelisk	χ^2, $P < 0.05$	Progeny on bread wheat cv. Shafir	χ^2, $P < 0.05$	IPO323	IPO95052	Progeny on bread wheat cv. Obelisk	χ^2, $P < 0.05$	Progeny on durum wheat cv. Inbar	χ^2, $P < 0.05$
			n = 74		*n* = 87				*n* = 99		*n* = 58	
AvrStb6	+	–	42:32	1.35	48:39	0.93	+	–	53:46	0.50	28:30	0.69
mat	*mat1-1*	*mat1-2*	41:33	1.11	47:40	0.56	*mat1-1*	*mat1-2*	55:44	1.22	31:27	0.28
ag-0006	B[a]	A[a]	36:38	0.05	52:35	3.32	B	A	43:56	1.71	28:30	0.69
mt-SSR	B[b]	A[b]	19:55	17.51*[c]	0:87	87.00*	B	C[b]	99:0	99.00*	43:15	13.52*[d]

Summary of segregation ratios for the nuclear markers for avirulence (*AvrStb6*), mating type (*mat*), and the microsatellite ag-0006, as well as the mitochondrial microsatellite marker mt-SSR in four populations derived from crosses between *Z. tritici* isolate IPO323 (adapted to bread wheat) with isolates IPO94269 (adapted to bread wheat) or IPO95052 (adapted to durum wheat) on three wheat cultivars with different modes of resistance (cv. Obelisk, which is generally susceptible to *Z. tritici* isolates derived from bread wheat; cv. Shafir, which carries *Stb6* (ref. [9]); and cv. Inbar, which is a tetraploid durum wheat cultivar that is resistant to virtually all *Z. tritici* from bread wheat, including IPO323 and IPO94269, but susceptible to most *Z. tritici* isolates from durum wheat, including IPO95052 (refs [4,39])). Asterisk, segregation ratios that differed significantly (χ^2 test, $P < 0.0001$) from a 1:1 ratio. [a]A and B represent the different alleles of ag-0006 in the parental isolates. [b]A, B, and C represent the different alleles of mt-SSR in the parental isolates. [c]Both parents have an expected equal chance of paternal and maternal parenthood, but the skewed segregation of mt-SSR is probably caused by competition of both isolates during pathogenesis on the susceptible cv. Obelisk, similarly to results in Fig. 3a as well as Fig. 2, in which both isolates were inoculated on cv. Taichung 29. [d]The expected segregation ratio is 0:58, but durum wheat cv. Inbar atypically allows pycnidia production by some bread wheat isolates, such as IPO323 (ref. [39]).

unexpected sexual reproduction between IPO323 and IPO94269 on cv. Shafir, despite the presence of *AvrStb6* in the avirulent parent IPO323 (Table 1; additional information in Supplementary Note). Sexual reproduction was further confirmed by crossing IPO323 and IPO95052 on the cvs. Obelisk or Inbar, which are susceptible to IPO323 and resistant to IPO95052 or vice versa, respectively (Table 1). We analyzed the four progenies with three nuclear markers (the avirulence gene *AvrStb6*; the mating-type alleles *mat1-1* or *mat1-2*; a random nuclear simple-sequence repeat (SSR) marker) and a mitochondrial SSR marker (mt-SSR)) and concluded that IPO323, despite its avirulence, underwent sexual reproduction with isolates

IPO94269 or IPO95052 (Table 1 and Supplementary Fig. 7). Thus, although IPO323 could not infect cvs. Shafir and Inbar, it completed a sexual cycle, thereby maintaining *AvrStb6* in subsequent populations (Fig. 2 and Supplementary Fig. 8). Moreover, crosses between sexually compatible *Z. tritici* strains did not fail unless both parents were avirulent (Supplementary Table 3). Notably, IPO323 was the exclusive paternal donor in the cross with the virulent isolate IPO94269 on cv. Shafir but swapped to the exclusive maternal—and virulent—donor in crosses with the avirulent isolate IPO95052 on cv. Obelisk, as shown by the mt-SSR marker, which is inherited only maternally (Table 1), as well as on cv. Taichung 29 (Fig. 3a). Thus,

Table 2 | Nonmendelian inheritance of resistance to azoxystrobin in ascospore progeny populations of *Z. tritici*

a

Rate	IPO03001 [R] × IPO03003 [S]		IPO03002 [R] × IPO03005 [S]		IPO04011 [R] × IPO04001 [S]	
	Total	% germination	Total	% germination	Total	% germination
0	630	93	856	15	2,108	49
1/32	85	89	390	48	709	64
1/16	188	92	183	78	556	100
1/8	191	76	135	88	607	100
1/4	237	100	166	88	336	99
1/2	496	99	105	93	349	100
Full dose	186	96	NA	NA	512	100
Total	2,013		1,835		5,177	

b

			Percentage resistant in PCR			
Rate	First round	Second round	First round	Second round	First round	Second round
0	100	100	0	33	100	38
1/32	100	100	98	49	96	71
1/16	100	100	100	100	65	98
1/8	100	100	100	98	100	100
1/4	100	100	96	98	100	100
1/2	100	100	92	92	100	100
Full dose	*	100	*	100	*	100
Double dose	*	100	*	100	*	100

[a]The percentage of strobilurin-resistant progeny was determined by monitoring the germination of 9,025 ascospores originating from 21 in planta crosses between strobilurin-resistant and strobilurin-sensitive *Z. tritici* isolates on seedlings of wheat cv. Taichung 29 that were preventively treated with six doses of Amistar and then discharged onto water agar amended with 1 p.p.m. (minimum inhibitory concentration value) Amistar normalized to germination frequencies of 15,975 ascospores from the same crosses that were discharged onto unamended water agar. [b]The percentage of strobilurin-resistant progeny was determined by a strobilurin-sensitivity PCR screen in 42 *Z. tritici* progenies. Crosses for full and double doses in the first round were not performed, as indicated by asterisks.
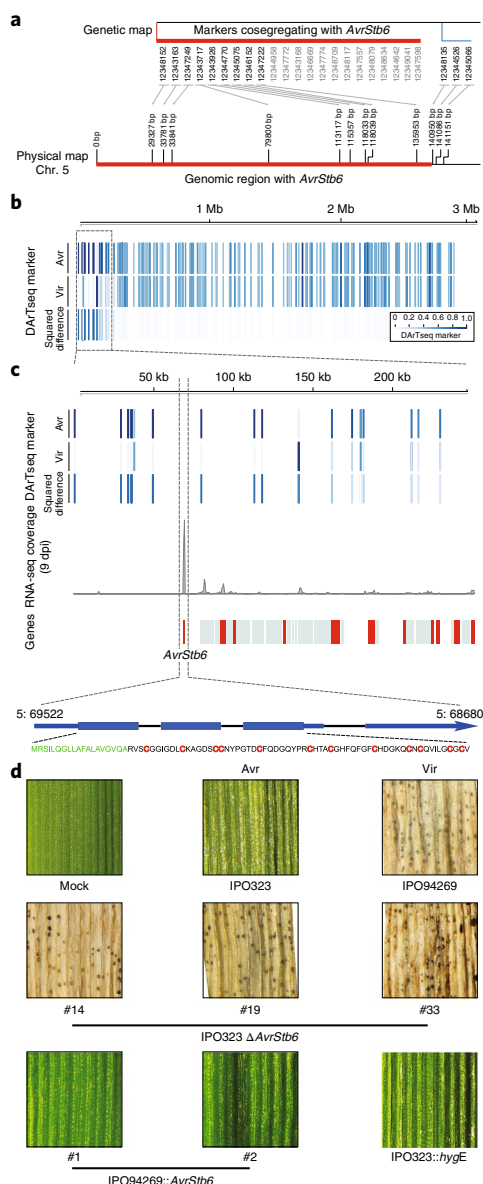
Fig. 1 | Cloning of *AvrStb6* in *Z. tritici* isolate IPO323. a, Genetic and physical maps of a tip of chromosome (chr) 5. A cluster of 22 DArTseq markers fully cosegregating with *AvrStb6* is highlighted in red. Markers with nonunique mapping are in gray, and flanking markers are in blue. Marker locations (bp) are indicated by arrows, and the genomic region bearing *AvrStb6* is indicated in red. **b**, Genomic locations of DArTseq markers on chromosome 5 are indicated by colored lines. The dashed rectangle highlights the only polymorphic region characterized by high squared differences of the fractions of DArTseq markers in avirulent (Avr) or virulent (Vir) progeny (color coded; scale 0–1). **c**, Magnification of the first 250 kb on chromosome 5 with the genomic locations of the DArTseq markers. The positions of predicted genes and genes encoding secreted proteins are indicated by gray and red bars, respectively. RNA-seq reads[21] indicate a single highly expressed gene, designated *AvrStb6*, encoding a secreted cysteine-rich effector protein. The blue line indicates exon–intron structure, with coding regions shown with extended line width. Amino acids in green indicate the predicted signal peptide, and cysteines are shown in red. Dpi, days postinfection. **d**, Phenotyping of *Z. tritici* on cv. Sharif (carrying *Stb6*). Top, mock and wild-type *Z. tritici* isolates IPO323 and IPO94269. Middle, independent knockouts of *AvrStb6* in IPO323 are virulent. Bottom, two independent introductions of *AvrStb6* in virulent strain IPO94269 (#1 and #2) are avirulent. A strain (IPO323::*hyg* E) with an ectopic integration of the deletion construct in IPO323 is similar to the wild type.
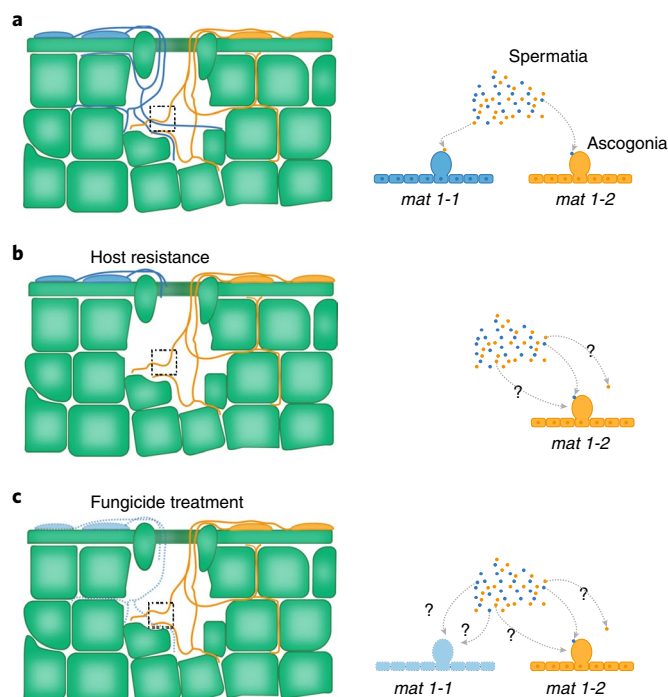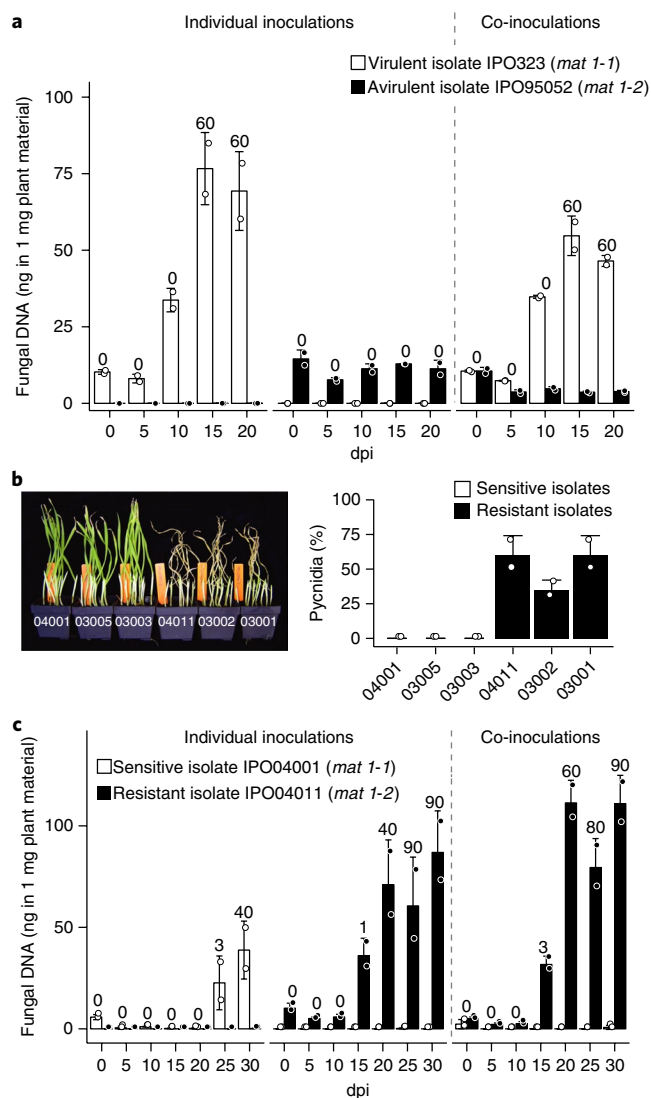

Fig. 2 | Sex in *Z. tritici*. The fungus has a heterothallic bipolar mating system. Each strain has a unique mating type, either *mat1-1* (blue) or *mat1-2* (orange). When both strains infect the same host, they produce female (ascogonia) and male (microconidia or spermatia[36,37]) reproductive organs. Both strains have equal chances of maternal or paternal parenthood. Owing to heterothallism, *mat1-1* ascogonia are fertilized exclusively by *mat1-2* spermatia, and vice versa. **a**, Optimal conditions for two pathogenic strains. **b**, An avirulent strain (*mat1-1*, blue) encounters biotic stress on resistant wheat, despite penetration[38]. The virulent strain (*mat1-2*, orange) colonizes the mesophyll. Biotic stress decreases the biomass of the avirulent strain but allows the production of spermatia. Owing to EPP, ascogonia of the virulent strain are fertilized exclusively by the avirulent strain. Consequently, avirulence genes are transmitted to the progeny and distributed by airborne ascospores. **c**, The sensitive isolate (*mat1-1*, blue dotted line) is under abiotic stress, whereas the resistant strain (*mat1-2*, orange solid line) colonizes the host after strobilurin application. Abiotic stress decreases the biomass of the sensitive strain but allows the production of spermatia. Owing to EPP, mating is accomplished exclusively by fertilization of the ascogonia of the resistant strain. Consequently, all progeny carry the *cytb* gene with the p.Gly143Ala alteration (fungicide resistance), which is maternally transferred and further disseminated by airborne ascospores.

isolate IPO323 circumvents unfavorable host conditions (i.e., resistance) via sexual reproduction as a male partner, a mechanism that we denote exclusive paternal parenthood (EPP; Table 1 and Fig. 2). Hence, we conclude that host resistance is a biotic stress factor that modulates parenthood in fungal sex. Therefore, our data challenge the common belief[14,16] that avirulent individuals disappear from natural populations because they can neither infect nor reproduce on resistant hosts.

To generalize these observations, we considered fungicides as abiotic stress factors for *Z. tritici* and hypothesized that they result in EPP of sensitive strains. We used the strobilurin fungicide Amistar and the mitochondrial *cytb* resistance allele as the maternally inherited marker (Fig. 3 and Supplementary Fig. 9). Six *Z. tritici* field isolates originating from Germany and the Netherlands with equal pathogenicity, opposite mating types, and contrasting fungicide resistance were crossed in three sets (Table 2, Supplementary Table 6, Fig. 3,

Fig. 4 | Comparison of the EPP model and Leonard's model in an agricultural and natural scenario. a, In the agricultural scenario, the frequency of the resistance allele in the plant population is constant, because it is under the control of growers and not affected by selection pressures imposed by the pathogen. Comparison of the EPP model and Leonard's model demonstrates the decreased rate of virulence buildup. The dashed EPP line represents a different set of parameters and demonstrates a polymorphism in the pathogen population (parameters of the model representing various fitness costs and allele frequencies in Supplementary Note). **b,** In the natural scenario, the resistance-allele frequency is dynamic and controlled by selection pressures in the system. Comparison of Leonard's model with the EPP model demonstrates the possible stability of the internal equilibrium point in the EPP model as well as the instability of the corresponding internal equilibrium point in Leonard's model (different parameter sets in Supplementary Note).
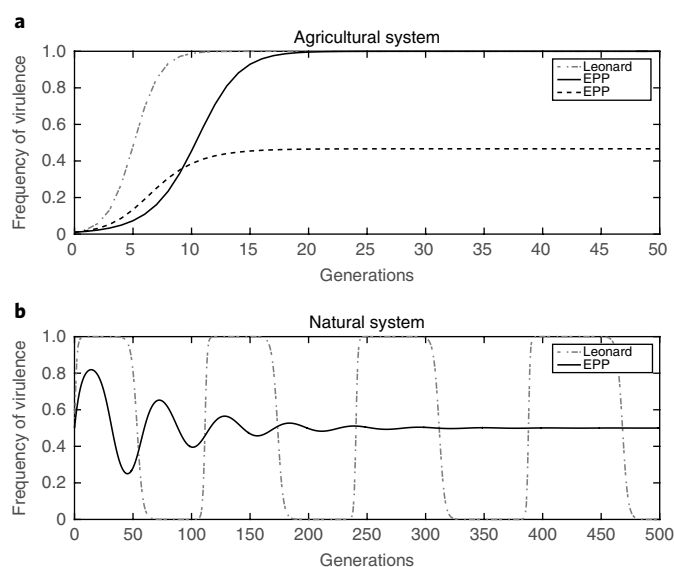

Fig. 3 | Inoculation and mating/competition assays with *Z. tritici*.
a, Quantitative biomass detection of isolates IPO323 (virulent) and IPO95052 (avirulent) and their co-inoculations on the bread wheat cv. Taichung 29 at 0, 5, 10, 15, and 20 dpi (averages of two independent experiments; error bars, s.d.). Percentage leaf area covered by pycnidia at each time point is shown as numbers over bars. **b**, Fungicide sensitivity screen at 20 dpi. Plants of wheat cv. Taichung 29 treated (48 h before inoculation) with the full recommended dose of the strobilurin Amistar (active ingredient azoxystrobin) and inoculated with the sensitive isolates 04001, 03005, or 03003 and with the resistant isolates 04011, 03002, or 03001 (right, percentage pycnidia on the basis of visual observations; averages of two independent experiments; error bars, s.d.). These treatments resulted in significantly different disease severities between the sensitive and resistant strains (both panels). **c**, Quantitative biomass detection of *Z. tritici* on cv. Taichung 29 after preventive treatment (48 h before inoculation) with the full recommended field rate of Amistar at 0, 5, 10, 15, 20, 25, and 30 dpi (three independent crossing experiments for the phenomenon; averages of two technical replicates; error bars, s.d.). Plants were inoculated with the sensitive isolate IPO04001, the resistant isolate IPO04011, or both (co-inoculations). Percentage leaf area covered by pycnidia at each time point is shown as a number over each bar.

Supplementary Fig. 10 and Supplementary Note). We produced 42 progenies under various concentrations of Amistar (Table 2) and determined the percentage of resistant ascospores through

either visual observation (9,025 ascospores) or PCR assays on 2,100 progeny isolates (50 per cross; Table 2 and Supplementary Note). Despite the use of fungicides, we confirmed sexual reproduction for all crosses (Table 2 and Supplementary Fig. 11). Sensitive strains were outcompeted in each crossing assay (Fig. 3 and Supplementary Fig. 10). Under normal and double azoxystrobin concentrations, all progenies were entirely fixed for resistance in one generation (Table 2). Thus, Amistar application directs resistant and sensitive isolates into maternal and paternal parenthood, respectively, thereby leading to a rapidly increasing frequency of resistance alleles in the generated progenies. In conclusion, we observed that biotic and abiotic stresses may hamper or restrict host colonization but cannot preclude sexual reproduction, because male gametes (spermatia) presumably survive biotic and abiotic stresses (Fig. 2).

We developed a population-genetic model by incorporating EPP into Leonard's seminal model of GFG coevolution of a plant–pathogen system[23] (additional information in Supplementary Note). In this model, the plant has one locus with alleles for resistance and susceptibility, and the pathogen has a corresponding locus with alleles for avirulence and virulence. The proportion of each allele in a well-mixed population is modeled over time. In real-life cases, alleles often coexist in stable or cyclic polymorphisms; however, in Leonard's model, the frequency of resistance and virulence alleles in the respective population results in fixation of only one of the genes, and coexistence is not possible (Fig. 4). Hence, Leonard's model provides a theoretical framework to identify traits whose inclusion can result in stable or cyclic polymorphisms (such as having multiple pathogen cycles per plant cycle, including a seed bank,

or incorporating spatial structure)[24]. We explored the consequences of incorporating the EPP reproduction mechanism into Leonard's model under two scenarios: first, when the frequency of the plant alleles is constant, as can be assumed in an agricultural system, and second, when the frequency of the plant is free to vary, as occurs in a natural ecosystem. In the agricultural scenario, the frequency of virulence in the pathogen population increases more slowly when avirulent strains partake in sexual reproduction on resistant hosts (Fig. 4). Additionally, polymorphisms (in which the two alleles can coexist indefinitely) are possible, although unlikely (additional information in Supplementary Note); however, polymorphisms are not possible without the EPP mechanism[24]. The modeling results imply that resistance in crop cultivars would erode more slowly than previously thought and might have major consequences for the sustainability of disease control in crop production systems. In the natural scenario, our model (Fig. 4) shows stable or cyclic polymorphisms occurring across a wide range of parameter values (additional information Supplementary Note). We therefore showed that the presence of sex under biotic stress allows for the occurrence of stable polymorphisms simply as a result of the pathogens' genetic system. Moreover, the model confirms that when fungicide-sensitive strains partake in sexual reproduction, the mitochondrially inherited *cytb* resistance allele invades faster than any nuclear inherited fungicide resistance allele (data not shown).

The experimental data and our theoretical model provide explanations for practical observations. Slow decline of host resistance is commonly observed in the wheat–*Z. tritici* pathosystem, in agreement with the unanticipated but ubiquitous presence of *Stb6* in many old and contemporary wheat cultivars around the world[9,25] (Supplementary Table 1). Compared with the typical boom-and-bust cycle in the yellow rust pathogen *Puccinia striiformis*, resistance to septoria tritici blotch declined significantly more slowly over a period of 10 years in the UK[26]. Strobilurin fungicides were commercially introduced in 1996 and initially showed excellent control of a wide range of plant pathogens including *Z. tritici*. However, resistance appeared in 1998 for powdery mildew in wheat, caused by *Blumeria graminis*[27], and in 2002 for *Z. tritici*, which then occurred throughout Europe 1 year later; at present, strobilurin resistance is fixed in most *Z. tritici* populations[28,29]. A similar trend for strobilurin resistance dynamics has been observed in *Pseudocercospora fijiensis*, the banana black sigatoka fungus[30,31].

Plant-disease management relies primarily on host resistance or fungicide applications[32,33]. Therefore, our observations on fungal sex have broad relevance for developing resistant host varieties and shaping disease-control strategies. This relevance may extend beyond plant pathogens to human fungal pathogens such as *Aspergillus fumigatus*, for which sex probably also contributes to the development of new life-threatening resistance mechanisms[34,35].

URLs. We conclude that fungal sex is an underestimated aspect of disease control that requires much more attention. Reference genome, http://genome.jgi.doe.gov/Mycgr3/Mycgr3.home.html/.

Methods
Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41588-018-0052-9.

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Author contributions

G.H.J.K., P.J.G.M.d.W., B.P.H.J.T., M.F.S., M.A.d.W., and C.W. designed the study. S.B.W., S.B.M., C.D.-T., E.Z., L.A., and E.C.P.V. performed the crossing assays and collected and analyzed the genotypic and phenotypic data. T.A.J.v.d.L., H.J.S., and L.A. generated the genetic linkage maps, and H.J.S. and L.A. fine mapped *AvrStb6*. L.A., A.M.G., H.A.Y.G., R.M., and H.J.G.M. performed *AvrStb6* candidate analyses and additional genome analyses, and A.M.G. and L.A. performed the functional analysis of *AvrStb6*. H.A.Y.G. and M.F.S. analyzed the genomic sequences and reannotated the IPO323 and IPO94269 genome sequence. G.H.J.K. and F.v.d.B. conceptualized the EPP model, and F.v.d.B., R.M.-S., V.A.-C., and J.H. mathematically developed the EPP model. G.H.J.K. wrote the paper with substantial input from S.B.W., B.P.H.J.T., H.J.G.M., M.F.S., F.v.d.B., and J.H. G.H.J.K. coordinated the project.

Competing interests

The authors declare no competing financial interests.

Additional information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41588-018-0052-9.

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Methods

Primer development and PCR conditions. To determine azoxystrobin sensitivity or resistance among generated *Z. tritici* ascospore progenies, we developed a mismatch amplification mutation assay (MAMA)[40] based on part of the cytochrome *b* (*cytb*) gene. Primers were designed with a mismatch on the penultimate nucleotide, and the ultimate nucleotide was at position 143 of *cytb*. The primer set used to specifically amplify a DNA fragment in sensitive isolates comprised the sense primer StrobSNP2fwd (5′–3′ (404–428)) with a mismatch of T instead of G at nucleotide 427 of *cytb* and the antisense primer StrobSNP1rvs (5′–3′ (1024–1043)). The primer set used to specifically amplify a DNA fragment in resistant isolates comprised the antisense primer StrobSNPrcF7 (5′–3′ (428–453)) with a mismatch of T instead of G at nucleotide 429 and the sense primer StrobSNPrcR1 (5′–3′ (152–173)). For the MAMA and mating-type PCR assays, 1 µl and 0.5 µl of DNA, respectively, were used.

Mating-type PCR primers and thermal-cycling conditions were as previously described[20]. Amplicons were analyzed on 1.2% agarose gels with 25-µl aliquots of the PCR products. PCRs to amplify SSRs comprised a 20-µl volume containing 20 ng DNA, 2 µl 10× PCR buffer with MgCl₂, 2 µl each forward and reverse primers (2 µM), 0.8 µl dNTPs (5 mM), 0.2 µl Taq DNA polymerase (5 U/µl), and sterile double-distilled water. The thermal cycling was as follows: cycle 1, 94 °C for 2 min; cycle 2 (repeated 12 times), 94 °C for 30 s, then 66 °C minus 1 °C per cycle for 30 s, then 72 °C for 30 s; cycle 3 (repeated 27 times), 94 °C for 30 s, then 53 °C for 30 s, then 72 °C for 30 s; cycle 4, 72 °C for 7 min, then cooling to 10 °C. Fragments were separated on a Mega-Gel Dual High-Throughput Vertical Electrophoresis Unit (CBS Scientific) with 6% nondenaturing acrylamide gels stained with ethidium bromide during the run.

To monitor the biomass of isolates in crossing and infection assays, we designed specific TaqMan probe/primer combinations for quantitative PCR (qPCR) based on the *mat1-1* and *mat1-2* idiomorph sequences of the two reference *Z. tritici* isolates IPO323 and IPO94269, respectively[20]. The primers used to specifically amplify DNA fragments in *mat1-1* isolates were Mmat1F3/Mmat1R3, with FAM fluorescent probe IP3, and the primers used to specifically amplify DNA fragments in *mat1-2* isolates were Mmat2F7/Mmat2R7, with YY fluorescent probe 2P4. Both quantitative real-time amplifications were performed in a single PCR on an Applied Biosystems 7500 Real-time PCR System. The total reaction volumes were 25 µl, including 3 µl DNA, 12.5 µl Premix Ex Taq (2×) (TaKaRa), 1 µl each forward and reverse primers (6 µM), 0.67 µl of each probe (5 mM), 0.5 µl ROX Reference Dye II (50×), and 8.33 µl ultraPURE nuclease-free water (Gibco). Thermal cycling was as follows: cycle 1, 50 °C for 2 min; cycle 2, 95 °C for 10 min; cycle 3, (repeated 39 times), 95 °C for 15 s, then 60 °C for 20 s. Results were analyzed with Sequence Detection Software version 1.2.3 (Applied Biosystems). Standard curves from serial dilutions of known concentrations of pure fungal DNA from the six parental isolates plus the DNA from the reference isolates (Supplementary Table 6) yielded highly similar Ct values. Therefore, serial dilutions of DNA from isolates IPO323 and IPO94269 were included in each TaqMan PCR run to calculate the unknown concentrations of fungal DNA in inoculated wheat seedlings. The standard curves had very high R^2 values (0.990–0.996) for all data points from 3 pg to 30 ng; therefore, Ct values within this range were deemed reliable (data not shown). All probes and primers used are listed in Supplementary Table 7.

Generation and analyses of segregating *Z. tritici* populations. *Crossing assays.* We used an in planta crossing protocol for all mating assays[6]. For mapping, we extended the existing *Z. tritici* mapping population IPO323/IPO94269 to 400 progeny isolates and the IPO323/IPO95052 population to 165 progeny isolates by manually collecting individual ascospores. For the EPP–biotic stress validation, we independently performed six crosses between avirulent and virulent isolates (IPO323, IPO94269, and IPO95052) on five wheat cultivars (Obelisk, Shafir, Taichung 29, Inbar, or Volcani 447) in multiple (two or more) biological replications. In addition, we used eight isolates in 19 crosses on nine wheat varieties (seven bread wheat and two durum wheat) and one barley accession (Supplementary Tables 2 and 3) to test the occurrence of sex despite one of the parents being avirulent. For the EPP–abiotic stress validation, we conducted 42 crosses between three sets of fungicide-resistant and fungicide-sensitive isolates on cv. Taichung 29 (Fig. 3, Supplementary Figs. 8 and 10, and Supplementary Table 6). SSR genotyping was routinely used either to confirm that segregating populations resulted from the applied parental isolates (Table 1 and Supplementary Figs. 7 and 11) or to determine the genotype of asexual fructifications that appeared in crossing assays (Supplementary Figs. 12 and 13). Populations were maintained at –80 °C (ref. [41]) for further detailed analyses including DArTseq as well as MAMA, diagnostic PCRs to determine mating-types[20] and maternal/paternal contributions to sexual development, sequencing/phenotyping to determine virulence or avirulence in progeny and wild-type strains (Tables 1 and 2, Supplementary Tables 4 and 5, and Supplementary Fig. 8), and qPCR (Fig. 3 and Supplementary Figs. 8 and 10).

Phenotyping. We prepared inoculum by following published procedures[19] and performed seedling assays at growth stage 11 or 12 (ref. [42]) either by painting a spore suspension with a soft brush (mapping populations) or by atomizing a spore suspension onto potted seedlings that were placed at the perimeter of a circular rotary table housed in an inoculation cabinet, rotating at 15 r.p.m., and equipped with interchangeable atomizers and a water-cleaning device to avoid cross-contamination between isolates (all other assays). Infected plants were incubated in transparent plastic bags for 48 h at 100% RH in the aforementioned greenhouse. Disease severity was assessed at 21 dpi on the basis of necrosis and pycnidial development, estimated as the percentage of affected tissue of the total primary leaf area of individual seedlings. After these procedures, we screened 190 IPO323/IPO94269 offspring isolates, partly in three independent replicates (81 isolates) or single tests (Supplementary Fig. 14) with the parental strains as controls, on cv. Shafir carrying *Stb6* and the susceptible control cv. Taichung 29.

Genetic mapping. Fungal genomic DNA was isolated with a standard CTAB–chloroform protocol. The parents and offspring ($n = 282$) of the *Z. tritici* mapping population (IPO323/IPO94269)[4,12] were assayed, of which 171 isolates showed distinct avirulence/virulence phenotypes on cv. Shafir. We used DArTseq, a GBS method that combines Diversity Array Technology and next-generation sequencing platforms[43]. In total 5,392 polymorphic DArTseq markers in *Z. tritici* isolates were obtained. Marker sequences (maximum of 69 nt) were placed on the *Z. tritici* reference genome (Fig. 1) by using NCBI BLASTn (megablast)[44] and visualized with the GViz package[45] (Fig. 1). Multimapping markers were placed on the genome at the best position only if there was a considerable difference in bit scores (difference ≥5).

For fine mapping, we sorted the 5,392 generated DArTseq markers according to their discrimination power for avirulent/virulent isolates by calculating the squared differences of genotype frequencies, then selected 60 DArTseq markers linked with avirulence. These markers were sorted into a genetic linkage map in JoinMap 4 software with settings LOD ≥3 for grouping and the maximum-likelihood mapping option for linkage-group generation[46]. Because the segregation of avirulence fit the model of single-gene inheritance[18] (Supplementary Fig. 14), we converted phenotypic data to an appropriate marker (*AvrStb6*) by using scoring codes that are required for JoinMap and integrated them in the mapping procedure.

Offspring isolates with >10% missing genotypic values were removed from the analysis. Moreover, isolates without recombination near the virulence or avirulence locus, and eight isolates showing discrepancies between the genotyping and phenotyping, were excluded from the analysis. To delimit the physical region containing *AvrStb6*, we performed a graphical mapping approach using the recombinant offspring isolates and clustered the markers that cosegregated with *AvrStb6* into bins, with the marker order estimated by JoinMap as a reference (Supplementary Fig. 15). The generated genetic linkage map was compared to the IPO323 reference genome sequence by aligning the DArTseq data to determine the physical position of *AvrStb6*.

Gene annotation. We performed gene annotation on the *Z. tritici* reference genome for isolate IPO323 (ref. [4]; URLs) by using the Maker2 pipeline[47], combining ab initio protein-coding-gene evidence from SNAP[48], Augustus[49], and GeneMark-HMM[50]. Additionally, Maker2 was provided with protein alignments to 35 predicted fungal proteomes, *Z. tritici* reference gene models annotated by the Joint Genome Institute (JGI)[4], and transcriptome data (assembled transcripts and splice junctions) derived from two previously published RNA-seq datasets[21,51]. For gene annotation, RNA-seq data (single-end) were mapped to the *Z. tritici* reference genome with TopHat (version 2.0.13) (--min-intron-length 20 --max-intron-length 2000 --max-multihits 5)[52]. *Z. tritici* transcripts were assembled with Cufflinks[53]. Gene models predicted with Maker2 were manually evaluated and refined[54], for example by excluding protein-coding genes <60 aa or lacking a starting methionine.

Identification of effector candidates. Gene expression, expressed as fragments per kilobase of exon per million fragments mapped (FPKM), during wheat colonization for newly predicted protein-coding genes was inferred with Cuffdiff (version 2.2.1)[53]. Similarly to previous observations[21,55], the third replicate of the RNA-seq experiment of Rudd et al.[21] behaved differently and was therefore excluded from all further analyses. Pairwise log₂ fold expression changes as well as multiple-testing-corrected *P* values ($P < 0.05$) were inferred for in planta RNA-seq samples compared to CDB[21]. N-terminal secretion signals were predicted in all proteins by using SignalP (version 4.1)[56]. Protein domains were predicted with InterProScan[57].

Functional analyses of *AvrStb6*. *Strains, media, and growth conditions.* *Z. tritici* strains IPO323 and IPO94269, which are avirulent and virulent, respectively, on cv. Shafir, were used as wild-type (WT) strains and recipient strains for gene deletion and ectopic expression (Supplementary Fig. 16). The WT and all deletion strains were kept at –80 °C and were recultured on potato dextrose agar (PDA) (Sigma-Aldrich) at 15 °C as needed for experimentation. Yeast-like spores were produced in yeast glucose broth (YGB) medium (yeast extract, 10 g l⁻¹; glucose, 30 g l⁻¹) after incubation in an orbital shaker (Innova 4430, New Brunswick Scientific) at 15 °C. For in vitro expression analyses in *Z. tritici* blastospores, we used YGB and MM[58] under similar conditions, whereas we adjusted the conditions in YGB to 25 °C for expression in mycelium. *Escherichia coli* DH5α was used for

general plasmid transformation, and *Agrobacterium tumefaciens* strain AGL-1 was used for all fungal transformations.

Fungal transformation. All transformations were performed with *A. tumefaciens*–mediated transformation, as described previously[59,60]. Genomic DNA of stable transformants was extracted according to standard protocols[61]. For ectopic complementation, the same procedure was used with minor modifications, including the use of geneticin (250 µg ml⁻¹) for the selection of mutants.

RNA isolation and qRT–PCR. In vitro and in planta expression profiling of *AvrStb6* was performed with quantitative real-time PCR (qRT–PCR). For in planta analyses, wheat cv. Shafir was inoculated in triplicate with the WT isolates, as previously described[60], and leaf samples were collected at 7 h postinoculation, and subsequently at 1, 2, 4, 8, 12, 16, and 20 dpi. Samples were then flash frozen and ground in liquid nitrogen with a mortar and pestle. Total RNA was extracted with an RNeasy plant mini kit (Qiagen) from either ground leaves or fungal biomass produced in YGB. DNA contamination was removed with a DNAfree kit (Ambion). First-strand cDNA was synthesized from approximately 2 µg of total RNA primed with oligo(dT) with SuperScript III, according to the manufacturers' instructions. The resulting cDNA (1 µl) was used in a 25-µl PCR with a QuantiTect SYBR Green PCR Kit and was run and analyzed with an ABI 7500 Real-Time PCR System. The relative expression of each gene was initially normalized to the constitutively expressed *Z. tritici* beta-tubulin gene[62] and was then calculated on the basis of the comparative Ct method described previously[63] (Supplementary Fig. 2).

Pathogenicity assays and quantitative fungal biomass analyses. All assays were conducted as described above, with wheat cvs. Shafir and Taichung 29 (Supplementary Figs. 5 and 17). Disease development was monitored and recorded every 3 d, and leaves of cv. Taichung 29 were harvested at 2, 4, 8, 12, 16, and 20 dpi for qRT–PCR expression analyses and for qPCR fungal-biomass determination of all WT and transformed *Z. tritici* strains[20,64] (Supplementary Fig. 17). Genomic DNA was extracted from approximately 100 mg of infected leaves with a standard phenol–chloroform DNA extraction[61].

Generation of gene deletion and ectopic integration constructs. To generate the *AvrStb6* deletion construct, pKOZtAvrStb6, a multisite Gateway three-fragment vector construction kit was used. This procedure enabled the cloning of three fragments into the destination vector, which was compatible with the *A. tumefaciens*–mediated transformation procedure. A 2-kb upstream and downstream sequence of *AvrStb6* was cloned in pDONR P4-P1R and pDONR P2R-P3. The generated constructs along with pRM250 (ref. [13]) containing the hygromycin phosphotransferase (*Hph*) gene as a selection marker were cloned into the destination vector, pPm43GW, via the LR reaction. To make the *AvrStb6* ectopic integration construct (pZtAvrStb6.com), the full ORF of *AvrStb6*, including a 1,020-bp upstream stretch as the promoter and a 552-bp stretch downstream as terminator, were cloned into pDONR P221 (Invitrogen), thus generating p221-ZtAvrStb6.com. The p221-ZtAvrStb6.com as well as two entry vectors, pRM245 and pRM234 (ref. [13]), were used to clone these three fragments into the destination vector, pPm43GW, through the LR reaction.

Determining exclusive paternal parenthood. *EPP–biotic stress.* To determine parenthood in the conducted crosses, we analyzed four crosses (Table 1 and Supplementary Figs. 6 and 7), using four markers (*AvrStb6*, *mat*, ag-0006, and mt-SSR), and monitored fungal biomass development through qPCR (Fig. 3 and Supplementary Fig. 8).

EPP–abiotic stress. Strobilurin sensitivity was assayed in six strains (Supplementary Table 6) on potato dextrose agar (PDA) plates amended with kresoxim-methyl (BASF) and trifloxystrobin (Bayer CropScience), and minimal inhibitory concentrations (MICs) were determined for two different technical samples of the fungicides by spotting isolates on strobilurin-amended PDA plates. The concentrations of kresoxim-methyl were 0.0025, 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, and 1.0 p.p.m., and the concentrations of trifloxystrobin were 0.00025, 0.0005, 0.001, 0.0025, 0.005, 0.01, 0.025, 0.05, 0.1, and 0.25 p.p.m. All isolates were spotted in triplicate in a volume of 5 µl per spot at a concentration of 4 × 10⁵ spores ml⁻¹. As a positive control for growth, isolates were also plated on PDA amended with the strobilurin solvent (1% methanol). Plates were incubated at 18 °C in the dark for 10 d, after which MIC values were assessed. A test progeny was generated by crossing *Z. tritici* isolates IPO03001 and IPO03003 and analyzed on amended PDA plates and subjected to MAMA assays to demonstrate that both methods are congruous.

MIC values were determined for the six parental isolates (Supplementary Table 6) for the commercially available fungicide Amistar (Syngenta), containing the active ingredient azoxystrobin at 0.1, 1.0, and 10 p.p.m. We then determined which concentrations of azoxystrobin to use for infection and crossing assays by using in planta dose–response curves for the sensitive *Z. tritici* isolates treated with different preventive applications of azoxystrobin (250 g l⁻¹ azoxystrobin; 50% E.C.) on 10-d-old seedlings of cv. Taichung 29 that were preventively treated (48 h) with a track sprayer calibrated to deliver the recommended application of 1 l ha⁻¹

sprayed at a rate of 250 l ha⁻¹, with the following percentages of the full recommended dose: 0, 3.125, 6.25, 12.5, 25, 50, 100, and 200 (corresponding to fungicide solutions of 0, 0.03125, 0.0625, 0.125, 0.25, 0.5, 1, and 2 g azoxystrobin l⁻¹, respectively). We then inoculated plants with *Z. tritici* and recorded the percentages of leaf area covered by pycnidia at 20, 23, 26, and 29 dpi for dose–response curve experiments; at 20 dpi for infection assays; and at 0, 5, 10, 15, 20, 25, and 30 dpi for qPCR biomass monitoring over time (Fig. 3 and Supplementary Figs. 9 and 10). Finally, we used three sets of *Z. tritici* field isolates IPO03001/IPO03003, IPO03002/IPO03005, and IPO04001/IPO04011, with equal pathogenicity, opposite mating types and contrasting sensitivity to azoxystrobin (Table 2, Fig. 3 and Supplementary Fig. 9) for the generation of 42 in planta ascospore progenies, and monitored the fungal biomass development of each isolate in each crossing assay (Fig. 3 and Supplementary Fig. 10) individually and in pairwise mixtures on untreated and preventively treated (48 h, 100% azoxystrobin) seedlings of the wheat cv. Taichung 29. Leaf samples were collected at 0, 5, 10, 15, 20, 25, and 30 dpi and were immediately frozen in liquid nitrogen before storage at –80 °C until lyophilization, subsequent DNA extraction, and qPCR analyses. Two extractions were made from each sample (technical repeats), and the mean results were expressed in ng fungal DNA per mg dry-weight leaf material. A first set of 18 crosses was performed in seedlings of cv. Taichung 29 that were preventively treated (48 h) with Amistar at 0% (control), 3.125%, 6.25%, 12.5%, 25%, and 50% of the full dose. In a second set of 24 crosses, we repeated these conditions but added two concentrations; full dose (100%) and double dose (200%) (Table 2 and Supplementary Table 6). From 6 to 12 weeks after inoculation, material was harvested for ascospore discharge and collection[6]. Ascospores were isolated as much as possible from diverse locations within a plate or within several plates from each cross to obtain random ascospore progenies. Baseline germination frequencies on unamended WA plates for all 42 progeny sets (*n* = 15,975) and randomly selected ascospores were determined. Germination frequencies of the 24 ascospore progenies for the second series of crosses were also determined on WA amended with 1 p.p.m. of the active ingredient azoxystrobin (*n* = 9,025), and these frequencies were expressed as percentages relative to the mean of the control germination frequencies on unamended water agar. We evaluated the percentage of resistant offspring through 2,100 independent MAMA PCRs (Table 2).

Developing the new population genetics model. The model equations (Supplementary Table 8) were numerically solved in C⁺⁺. Output was plotted with the graphics package SigmaPlot. Calculations were performed by hand and checked in the package Maple to demonstrate that the frequency of virulence increased more slowly when the avirulent strain partakes in sexual reproduction, independently of the parameter values (additional data in Supplementary Fig. 18). We modeled the population-genetic consequences of this new observation by using an allele-frequency model, as introduced by Leonard[23].

Life Sciences Reporting Summary. Further information on experimental design is available in the Life Sciences Reporting Summary.

Data availability. All data are available and have been deposited in the NCBI GenBank database under accession number ACPE00000000 (ref. [4]), in the Gene Expression Omnibus database under accession number GSE54874 (ref. [51]), and as an NCBI BioProject under accession number PRJEB8798 (ref. [21]).

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Date: 29 November 2017

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▶ Experimental design

1. Sample size

   Describe how sample size was determined.

   We made 69 crosses in total (2 for extending mapping populations; 6 for the biotic EPP model, 19 for determining sex between isolates with differential pathogenicity responses and 42 for the abiotic EPP model). Each resulting in numerous ascospores that were analysed for each population. We monitored over 9,000 germination assays, ran 2,100 MAMA PCR assays for fungicide resistance in the progenies and for pathogenicity phenotyping usually over 10 progeny per cross. For knock-out strains we developed three indepedent homologous recombinant strains as well as several ectopic integration strains. The sample size were meeting the size required to demonstrate single gene inheritance (usually >25 progeny).

2. Data exclusions

   Describe any data exclusions.

   No data were excluded, but we included "not shown" statements (at P5L13; P7L13) where we indicate that the EPP model also demonstrates that mitochondrially inherited genes invade slower than nuclearly inherited genes (our focus is on the nuclear genes and their behaviour in the discovered and described EPP model) and on reliability of Ct values for qPCR, respectively.

3. Replication

   Describe whether the experimental findings were reliably reproduced.

   See above, all crosses were repeated at least twice and ascospores were indepedently harvested in the described discharge experiments. Phenotyping for pathogenicty - of KO Z. tritici strains - was with three biological repetitions and in three replicates.

4. Randomization

   Describe how samples/organisms/participants were allocated into experimental groups.

   Progenies from crosses were isolated at random from independant discharges. Phenotyping assays were fully randomized and statistical analyses were performed accordingly.

5. Blinding

   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

   na

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or the Methods section if additional space is needed).

n/a	Confirmed	
☐	☒	The <u>exact</u> sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☐	☒	A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly.
☐	☒	A statement indicating how many times each experiment was replicated
☐	☒	The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
☒	☐	A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☐	☒	The test results (e.g. *p* values) given as exact values whenever possible and with confidence intervals noted
☐	☒	A summary of the descriptive statistics, including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
☐	☒	Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

▶ Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

> Excel, Genestat and Matlab (presented in Supplementary Note Matlab code)

For all studies, we encourage code deposition in a community repository (e.g. GitHub). Authors must make computer code available to editors and reviewers upon request. The *Nature Methods* guidance for providing algorithms and software for publication may be useful for any submission.

▶ Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

> No unique materials used.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

> na

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

> na

b. Describe the method of cell line authentication used.

> na

c. Report whether the cell lines were tested for mycoplasma contamination.

> na

d. If any of the cell lines used in the paper are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

> na

▶ Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

> na

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

na