Prepublication:

Consequences of *in vitro* evolution on the virulence of the pathogen *Zymoseptoria tritici*

Jallet[1,2] AJ, Robert-Siegwald[1] G, Genissel[1] A

Author's affiliation:

[1] UMR BIOGER, INRA, AgroParisTech, Université Paris Saclay, Thiverval-Grignon, France

[2] Univ. Paris-Sud, Université Paris-Saclay, Orsay, France

Authors' email address: arthur.jallet@inra.fr, guillaume.rbt@gmail.com, anne.genissel@inra.fr

Corresponding author: Anne Genissel, anne.genissel@inra.fr, (33) 130815359

Abstract:

- All species are living in variable environments. The effect of changing environments should be more integrated to plant-pathogen studies to expend our knowledge on the evolutionary dynamic of plant-pathogen interactions.
- Following an experimental evolution *in vitro* during 500 generations with different temperature regimes, we investigated the virulence level of *Zymospetoria tritici* evolved lineages on 6 wheat cultivars and whole-genome sequenced 10 evolved lineages to seek accumulated mutations.
- Our study evidenced a decrease of virulence for several evolved lineages, depending on the host genotype. Our results evidenced trans-lineage segregating mutations in the genome, including synonymous and non-synonymous mutations in the secretome. Surprisingly, no loss of dispensable chromosomes occurred during the experimental evolution after 500 generations.
- These results suggest that abiotic environment can influence the dynamic evolution of the plant pathogen *Z. tritici*.

Key words: abiotic environment, genome integrity, de novo mutation, plant-pathogen evolution, secretome.

Introduction

Understanding how species are able to respond to environmental changes remains a challenge in Evolutionary Biology. In the quest to understand the dynamic arms race between plants and their pathogens, the effect of changing environments has been rarely considered, despite its ubiquitous effect on plant-pathogen evolution (Velasquez et al., 2018). Studies in laboratory controlled conditions hence represent a first step towards understanding the complexity of plant pathogens in the context of global change. In the present study our model is the ascomycete fungus *Zymoseptoria tritici,* a pathogen responsible for Septoria Tritici Blotch (STB), one of the most important foliar diseases of wheat (Fones and Gurr, 2015; O'Driscoll et al., 2014).

Knowledge on the complex molecular interactions between the wheat and this fungal pathogen is still growing (Kettles and Kanyuka, 2016). So far there are 21 known resistance genes and many more other QTLs with the continuous deployment of resistant cultivars in wheat by breeders (Brown et al., 2015), but investigations on the genetic basis of fungal pathogen virulence have been largely neglected (Bartoli and Roux, 2017; Genissel 2017). There has been no study on the genetic basis underlying virulence of *Z. tritici* but a few recent ones, advanced by NGS technologies which open new opportunities for non-model species. Since the publication of the first complete genome of *Z. tritici* (Goodwin et al., 2011), two main approaches are thus employed to dissect the genotype-phenotype link for the quantitative variation of virulence among *Z. tritici* populations: bi-parental QTL mapping using RADseq and Association Genetics using whole genome sequencing. Two QTL mapping studies have been published thus far, revealing QTLs (or genes) of large effect on chromosome 7 (Stewart et al., 2017; Meile et al., 2018). Genome-Wide Association Studies (GWAS) using a sample size > 100 individuals have also identified two effector proteins (gene Avrstb6 on chromosome 5 by Zhong et al., 2017; Hartmann et al., 2017). It is now becoming quite clear from these

studies that small secreted proteins are key components in the hide-and-seek evolutionary game between the pathogen and its host. In addition, each of these candidate effectors is located near transposable elements (TE) or in AT-rich regions. This might suggests that chromosomal rearrangements caused by TE are setting up the stage for rapid mutation accumulation in functionally relevant genes. Indeed despite the lack of evidence for their contribution to phenotypic variation many other effector genes are fast evolving and are located in regions with a high recombination rate (Poppe et al., 2015; Croll et al., 2015).

In this study we report the virulence level of evolved populations in the laboratory starting from a single clone, after being exposed to *in vitro* environment for many generations. We wanted to test the hypothesis that evolution *in vitro* for one year could affect the ability of the pathogen to invade its host. Pervasive transcriptome rewiring has been previously reported among the evolved strains, highlighting candidate genes which transcription level could be involved in temperature fluctuations adaptation. In addition, we observed more gene expression evolution at tips of core chromosomes as well as on accessory chromosome. In the present study, we used NGS to examine the genetic changes that could be linked to the phenotypic changes. Disease level of 18 evolved strains and the two founder clones (called MGGP01 and MGGP44) was assessed on six cultivars of wheat, which represent a large spectrum of resistance to STB. Main results highlight (*i*) a more frequent loss of symptoms for the lineages that evolved under fluctuating selection (*ii*) accessory chromosomes were not lost during the experimental evolution (*iii*) several mutations were identified among the evolved lineages and resulted in amino-acid changes, including in effector genes.

Materials and methods

Biological material

Z. tritici strains used in this study resulted from an experimental evolution, testing for three different temperature regimes: stable at 17°C, stable at 23°C and a fluctuating regime between these two temperatures. Evolved lineages and ancestral clones used in this study are described in **Figure 1**. The two ancestors correspond to the strains MGGP01 and MGGP44 isolated in 2010 from lesions on cultivar Caphorn and cultivar Apache, respectively. Both strains were collected at the same location (South of France, Auzeville-Tolosane) (43° 53' N - 1° 48' W). Each strain was stored in 30% glycerol at -80°C. For sequencing, 10 strains were used (both ancestors and four evolved lineages derived from each, see **Figure 1**). Since the experimental evolution was conducted at two different temperatures (17°C and 23°C), cell multiplication for NGS was performed at 17°C and 23°C, in 500mL of liquid Potato Dextrose Broth medium, for each of the 10 strains, in the same conditions as for the experimental evolution (Jallet et al., 2019). Prior to phenotyping, aliquots from -80°C stocks were multiplied on petri dishes using Potato Dextrose Agar medium, at 20°C.

For *in planta* experiments we used six bread wheat cultivars which are known to manifest different resistance level against STB in the French fields: Caphorn, Apache, Premio, Courtot, Obelisk and Taichung29 (**Figure 2A**).

Plant infection

Six days prior to inoculation fungal samples were multiplied *in vitro* on PDA medium at 20°C. Spores were diluted to a concentration of 10^7 cells per mL in distilled water, using repeated counting with microglass chambers (Kova Glasstic R Slide 10). Tween 20 was added to the cell suspension to favour the fungal adherence at the leaf surface (0.1% v/v). One negative control (a leaf inoculated with water), was used within each pot, to check for the quality of leaf

development and verify the absence of cross-contaminations of fungal cell suspension between leaves.

We planted 5 seeds, negative control included, per fungal genotype for each experiment. Five independent replicated experiments were conducted, thus totalizing 25 plants for each plant genotype-by-fungal genotype interaction. Before and after inoculation, plants were maintained in a large growth chamber (16h/8h day/night, 22°C/18°C, 80%/95% relative humidity). Leaf inoculation was performed on 14 days-old seedlings by painting cell suspensions onto a segment of 7 cm of the first true leaf. To enable fungal dispersion at the leaf surface, plants were maintained for 2.5 days at a higher humidity rate of 100%. At 12 days post inoculation (dpi), plants were watered with nutritive solution (*PlantProd* fertilizer). At 21 dpi, we monitored the quantitative variation of symptoms by visual inspection using binoculars. We annotated for each inoculated leaf the percentage of leaf area covered with lesions (pycnidia) (PLACL).

Genomic DNA extraction

DNA was isolated for 10 lineages (**Figure 1**). Since the experimental evolution was conducted at two different temperatures, cell suspension and whole genome sequencing were also conducted at 17°C and 23°C. Snap frozen lyophilized mycelium (LYOVAC^{TM}, Steris) was grinded and vortexed in 14mL Lysis buffer at 65°C and incubated for 30 minutes at 65°C. Then samples were gently mixed in 3M Sodium Acetate pH5.2, incubated on ice for 30min and centrifuged at 10,000G for 15min at 4°C. Supernatant was transferred onto 0.7 volume of cold absolute isopropanol. Samples were centrifuged at 6,000G for 10min at 4°C, and pellets were washed in 4mL of cold 70% ethanol. Air-dried pellets were re-suspended in 4.3 mL of Tris-EDTA. After RNAse treatment, nucleic acids were extracted using Phenol *chloroform. DNA*

precipitation was done using Ammonium Acetate 7.5M and absolute ethanol. Each sample was re-suspended in very low Tris-EDTA buffer.

Illumina sequencing and variant calling procedure

Genomic DNA was sequenced via the Illumina HiSeq 2000 (Genewiz, Leipzig). Short sequence reads were trimmed using Trimmomatic v0.32 (Bolger et al., 2014) and aligned to the masked reference genome (Goodwin et al., 2011), using BWA v0.7.15 (Li et al., 2009). Alignments were converted to BAM using SAMtools v0.1.4 (Li et al., 2009). The percentage of IPO323 chromosomes covered by reads for each sample was calculated via BEDtools genomecov (Quinlan and Hall, 2010).

In order to identify SNPs and short indels segregating among the different lineages used in this study, we extracted variants from each sample using the GATK v-3.3-1-0 (McKenna et al., 2010) HaplotypeCaller with the following parameters: Samples were pooled using GenomeAnalysisTK with the following parameters: -T genotypeGVCFs GVCF --variant_index_type LINEAR –max_alternated_alleles 4 -ploidy 1. Identified SNPs and short indels were filtered using VariationFiltration –filterExpression "QD < 2.0 || FS > 60.0 || MQ < 40.0". The impact of each variable site on annotated genes was estimated using SnpEff v 4.3 (Cingoolani et al., 2012), and variant effects on the secretome were obtained using SnpSift v4.3 (Cingoolani et al., 2012) and a bed file from Morais Do Amaral et al., 2012.

Statistics

To estimate the significance of symptom differences among the fungal lineages we analyzed symptoms independently for each wheat cultivar. We used the following mixed linear model with the R package *nlme:*

$D \sim B_i + S_j + B_i*S_j + RegREP(Vir(L)),$

where D is the disease symptoms, B_i is the fixed effects of the genetic background (i= MGGP01 or MGGP44), Sj is the fixed effect of the evolutionary status of fungal samples (j= ancestral, evolved at 17°C, evolved at 23°C or evolved under fluctuations), B_i*S_j the interaction term. For each genetic background, the three lineages per selection regime were included (see **Figure 1**). This level of replication was considered random (*RegREP*), and both virulence assay (Vir) and leaf (L) were nested ramdom effects within the regime replicate effect.

For each genetic background, we calculated the contrasts between the evolutionary status *Sj* effect using mutltcomp R package and FDR threshold at 5% (Benjamini-Hochberg, 1995).

Results

Quantitative variation of disease symptoms among wheat cultivars

Knowledge on the genetic basis of resistance level among wheat cultivars is incomplete (Brown et al., 2015). In the present panel comprising six cultivars, only 4 resistance QTLs have been identified so far: Stb4 and Stb11 in Apache (Ghaffari et al. 2011), Stb6 in Caphorn (Zhong et al. 2017), and Stb9 in Courtot (Chartrain et al., 2009) (**Figure 2B**).

Although phenotypic distribution of disease symptoms was sometimes dispersed (see for example boxplots of ancestors MGGP01 and MGGP44 on cultivar Premio), we still detected significant differences of symptom between ancestors and the evolved lineages.

Significant decrease of virulence of evolved lineages

For the *in vitro* regimes of evolution, the significant effects we detected were exclusively associated with a reduced ability to make symptoms. The decrease of symptom was highly dependent on the wheat cultivar and on the fungal genetic background (MGGP01 or MGGP44). The **Figure 3** illustrates the symptom distribution of each cultivar separately. For the most susceptible cultivars Obelisk and Taichung29, significant loss of symptoms was varying between regimes and background genotypes, but on both cultivars, the strains MGGP01 that evolved under fluctuation were much less virulent than the ancestor. For the cultivar Premio, lineages from MGGP01 genetic background that evolved under fluctuations made significantly less symptoms than their ancestor. Likewise, lineages from MGGP44 genetic background that evolved at 17°C made significantly less symptoms. For the cultivar Apache, we found that evolving under thermal fluctuations or at 17°C resulted in a significant decrease of symptoms for MGGP44 genetic background.

When comparing the two stable regimes of evolution, more significant loss of symptoms were found after evolution at 17°C rather than after evolution at 23°C (7 out 10 comparisons) (**Table 1**). Although not significant, two out of the three remaining comparisons displayed a similar tendency ($P \leq 0.1$) between these two selection regimes. The same pattern was observed when we compared the symptoms between fluctuation and 23°C selection regimes, with 8 out of 10 comparisons with a greater loss of symptoms after evolution under fluctuation rather than after evolution at 23°C (**Table 1**). This suggests that both evolution at 17°C and under temperature alternating between 17°C and 23°C affected the disease level more severely compared to a stable temperature of 23°C. In addition, while the fluctuating selection regime caused rarely significantly less symptoms the selection at 17°C (3 cases, all for genetic background MGGP01), the opposite pattern was not observed.

No loss of accessory chromosomes

We estimated the coverage along the whole genome to check whether the number of accessory chromosomes was variable after the experimental evolution *in vitro*. Our data suggest, despite visible polymorphism between the two genetic backgrounds, that there is no loss of accessory chromosomes among the sequenced lineages (**Figure 4**).

Pattern of mutation accumulation between the evolved lineages:

We observed on average 0.06% of the genome with mutations that were identified among the evolved lineages (**Table 2**). Since we used only one tool in our bioinformatics pipeline we have to be cautious with the interpretation of these results. Further bioinformatics investigation is required. Likewise, further validation using Sanger sequencing for a subset of the mutations will be helpful to confirm these results. Nonetheless the distribution of SNPs along the genome is rather peculiar with a repeated pattern between genotypes and regimes, for the most part (**Figure 5A**). For small insertion-deletions, the distribution of variable sites appeared to be more random (**Figure 5B**). A combined search of small scale with large scale mutations using tools dedicated to identifying structural rearrangements should provide more insights into the nature and the distribution of accumulated mutations during the experimental evolution.

Effect of mutation accumulation on the secretome.

Considering the predicted secretome annotation defined by Morais Do Amaral et al. (2012), we identified mutations within genes which are predicted proteins to play a key role by counteracting the host immune response. Our results show a few mutations accumulated in some of these genes, which are dispersed across the genome. **Figure 6** reports the total number of these mutations by category of functional effect of the nucleotide substitution, either located in exons (synonymous or non-synonymous), introns, 5 prime- and 3 prime-untranslated regions (UTRs) (no stop codon mutation were detected) (**Figure 6A** and **6B**). We see a large number

of non-synonymous mutations for both backgrounds (**A** and **B**) and also a large number of mutations in introns for MGGP44. While effectors are expected to cumulate variants due to positive or diversifying selection due the host immune system selection pressure, the observation of a greater number of non-synonymous than synonymous after *in vitro* evolution is rather surprising. In addition, we report the mutational effects of identified small insertion deletions that were also located in this set of coding regions (**Figure 6C** and **6D**). We detected a few disruptive effects of indels. For both backgrounds, we found an excess of intronic and frameshift mutations. In conclusion, first we need to validate the nature and location of these variants before pursuing in the quest of understand rather these mutations play a role for adaptive evolution during our experiment.

Discussion

This work represents the first attempt towards understanding the influence of the selection under variable environment on the virulence level of the fungus *Z. tritici,* using experimental evolution. Using a panel of six wheat cultivars which are gradually different for their resistance level against *Z. tritici*, our results support a loss of virulence although the phenotypic response between evolved lineages is often heterogeneous. Our results also suggest that a complex genetic basis is underlying the quantitative variation of pycnidiae produced by the pathogen, in agreement with former studies, also suggesting that many unknown molecular factors – most likely of small or moderate effect– contribute to the phenotype (Stewart et al., 2017). Due to the lack of power in our study we did not investigate the link between genotype and phenotype through association tests. However we were still interested to seek new mutations in the evolved lineages that could be of strong relevance regarding to their function. To reach this goal, we annotated all mutations in the secretome, and found a few sites and small indels that were variable between the evolved lineages and their ancestor. The nature of these

mutations (such as amino-acid substitutions or for example mutations disrupting the reading frame of genes) highlights the high potential of this fungal species for rapid evolution. Whether they are truly associated with the observed decrease of virulence remains to be elucidated. These new alleles were either unique to a lineage, or shared between lineages. Trans-lineage polymorphism is not an expected result if we consider that mutations accumulated following a random walk during the process of evolution.

A recent study evidenced a loss of entire chromosomes belonging to the dispensome after a few transfer of *Z. tritici in vitro* (Möller et al., 2018). The opposite result found in our experiment is rather surprising. Our analysis on genome reads thus require a more thorough approach by combining different bioinformatics tools such as Delly2, SNVator which can reveal large structural rearrangements (SV). These SV are well known in *Z. tritici* from studies comparing natural population samples. The extent of coverage difference along the genome between the two strains that were used as ancestors for our experimental evolution is indeed another illustration that SV are ubiquitous in *Z. tritici* populations. It is known that many rearrangements occur during meiosis (Croll et al., 2015; Fouché et al., 2018). In conclusion our results suggest that during mitosis this type of rearrangements may occur less frequently. An explanation for the discrepancy on the maintenance of the genome integrity between studies is the genotype effect. Maybe some strains are more prone than others to lose their accessory chromosomes during *in vitro* conditions. An alternative explanation we want to propose is the effect of the medium on which the fungus is fed during the course of the experiments. Indeed a recent paper where the authors compared the differences of transposable element expression between nutrient-rich and nutrient-deprived culture mediums (Fouche et al, preprint). These results suggest that culture medium can affect structural rearrangements driven by TE transpositional activity.

We focused this study on genes belonging to the secretome. However, many other variable sites were detected when the allele in evolved lineages were compared to the ancestor alleles. We cannot exclude that some of these mutations are also contributing to the decrease of the disease observed among the evolved lineages. First, all effector proteins are likely not yet annotated (including genes that could be present in our two ancestor but absent from the reference genome of the strain IPO-323 (Plissonneau et al., 2018)). Second, other mutations in the genome could affect the level of virulence. Whether these mutations are regulatory or coding, on effector genes or not, this suggest there might antagonistic pleiotropy for some genes that contribute both to the growth *in vitro*, and the development of the *pathogen in planta*. A former QTL study on *Z. tritici* identified several candidate genes with pleiotropic effect, affecting both melanisation and morphology (Lendenmann et al. 2016).

Conclusion

Overall, these results suggest that the abiotic environment can influence the course of evolution of the pathogen *Z. tritici* and thus is very important to consider for the management strategies to control the pathogen populations.

References

Bartoli C and Roux F, 2017 Genome-Wide Association Studies In Plant Pathosystems: Toward an Ecological Genomics Approach. Frontiers Plant Sci. 8:763.

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30: 2114–2120.

Brown JK, Chartrain L, Lasserre-Zuber P, Saintenac C. 2015 Genetics of resistance to Zymoseptoria tritici and applications to wheat breeding. Fungal Genet Biol. 2015 Jun; 79:33-41.

Cheng, C, Gao X, Feng B, Sheen J, Shan L, He P 2013. Plant immune response to pathogens differs with changing temperatures Nat Commun. 4:2530. doi: 10.1038/ncomms3530

Cingolani P, Platts A, Wang le L, et al. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. Fly (Austin). 2012 6:80–92. doi:10.4161/fly.19695

Croll D, Lendenmann MH, Stewart E, McDonald BA. 2015. The impact of recombination hotspots on
genome evolution of a fungal plant pathogen. Genetics 201:1213–28

Fones H, Gurr S. 2015. The impact of Septoria tritici Blotch disease on wheat: an EU perspective. Fungal Genet Biol 79: 3–7

Fouché S, Badet T, Oggenfus U et al. preprint Stress-driven transposable element de-repression dynamics in a fungal pathogen

Fouché S, Plissonneau C, McDonald BA, Croll D. 2018 Meiosis Leads to Pervasive Copy-Number Variation and Distorted Inheritance of Accessory Chromosomes of the Wheat Pathogen Zymoseptoria tritici. Genome Biol Evol. 10(6):1416–1429.

Genissel A, Confais J, Lebrun M-H, Gout L. 2017 Association Genetics in Plant Pathogens: Minding the Gap between the Natural Variation and the Molecular Function. Front Plant Sci. 8: 1301.

Goodwin SB, Ben M'Barek S, Dhillon B, Wittenberg AHJ, Crane CF, Hane JK et al. 2011. Finished genome of the fungal wheat pathogen Mycosphaerella graminicola reveals dispensome structure, chromosome plasticity, and stealth pathogenesis. PLoS Genet 7: e1002070–17.

Hartmann FE, Sánchez-Vallet A, McDonald BA, Croll D. 2017. A fungal wheat pathogen evolved host specialization by extensive chromosomal rearrangements. The ISME Journal 11: 1189.

Jallet AJ Le Rouzic A A Genissel (20 August 2019). Evolution and plasticity of the transcriptome under temperature fluctuations in the fungal plant pathogen Zymoseptoria tritici. bioRxiv 720510.

Kettles GJ, Kanyuka K. 2016. Front Plant Sci. 2016; 7:508. Dissecting the Molecular Interactions between Wheat and the Fungal Pathogen Zymoseptoria tritici.

Li H, Durbin R. 2009 Fast and accurate short read alignment with Burrows–Wheeler transform, Bioinformatics, 25:14, 1754–1760

Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009 25:2078–2079.

McKenna, A et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 2010 20 : 1297-303.

Meile L., Croll D., Brunner P. C., Plissonneau C., Hartmann F. E., Mcdonald B. A., & Sanchez-Vallet A. (2018). A fungal avirulence factor encoded in a highly plastic genomic region triggers partial resistance to septoria tritici blotch. New Phytologist, 219(3), 1048–1061.

Morais do Amaral A, Antoniw J, Rudd JJ, Hammond-Kosack KE. 2012. Defining the predicted protein secretome of the fungal wheat leaf pathogen Mycosphaerella graminicola. PLoS One 7: e49904.

O'Driscoll, A Kildea S, Doohan F, Spink J, Mullins E. 2014 The wheat–Septoria conflict: a new front opening up? Trends in Plant Science, Volume 19, Issue 9, 602 – 610

Plissonneau C, Hartmann FE, Croll D. 2018 Pangenome analyses of the wheat pathogen Zymoseptoria tritici reveal the structural basis of a highly plastic eukaryotic genome. BMC Biol.16:5.

Poppe S, Dorsheimer L, Happel P, Stukenbrock EH. 2015. Rapidly evolving genes are key players in host specialization and virulence of the fungal wheat pathogen Zymoseptoria tritici (Mycosphaerella graminicola). PLoS Pathogens 11: e1005055.

Quinlan AR, Hall IM, BEDTools: a flexible suite of utilities for comparing genomic features, Bioinformatics. 2010 26: 841–42.

R Core Team. 2014 R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing, Available at http://www.R-project.org/.

Stewart E. L., Croll D., Lendenmann M. H., Sanchez-Vallet A., Hartmann F. E., Palma-Guerrero J. et al. 2017. QTL mapping reveals complex genetic architecture of quantitative virulence in the wheat pathogen Zymoseptoria tritici. Molecular Plant Pathology, 19, 201–216

Upson JL, Zess EK, Bialas A, Wu C-h and Kamoun S 1 The coming of ageof EvoMPMI: evolutionary molecular plant-microbe interactions across multiple timescales.Current opinion in plant biology 44:108-116.

Velasquez AC, Castroverde CD, He SY 2018 plant–pathogen warfare under changing climate conditions Current Biology 28, R619–R634

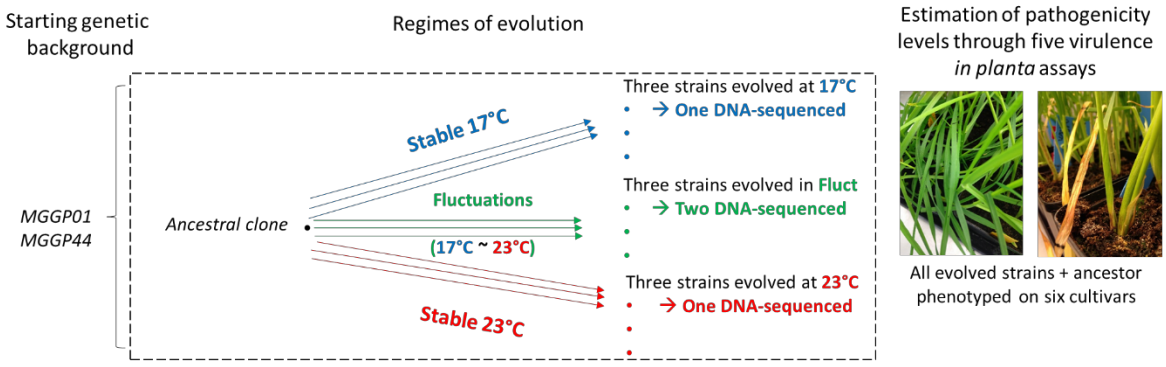


Figure 1 - Experimental evolution design prior to in planta phenotyping

From each of the two clonal ancestors (MGGP01 and MGGP44), three replicates per regime evolved in the following conditions: at 17°C, at 23°C and under temperature fluctuations. All evolved lines plus the two ancestors were phenotyped on six wheat cultivars by measuring their ability to cause symptoms *in planta*. In addition, the two ancestors and four evolved lineages from each of them (one Stable_17°C, one Stable_23°C and two fluctuating) were whole-genome sequenced.

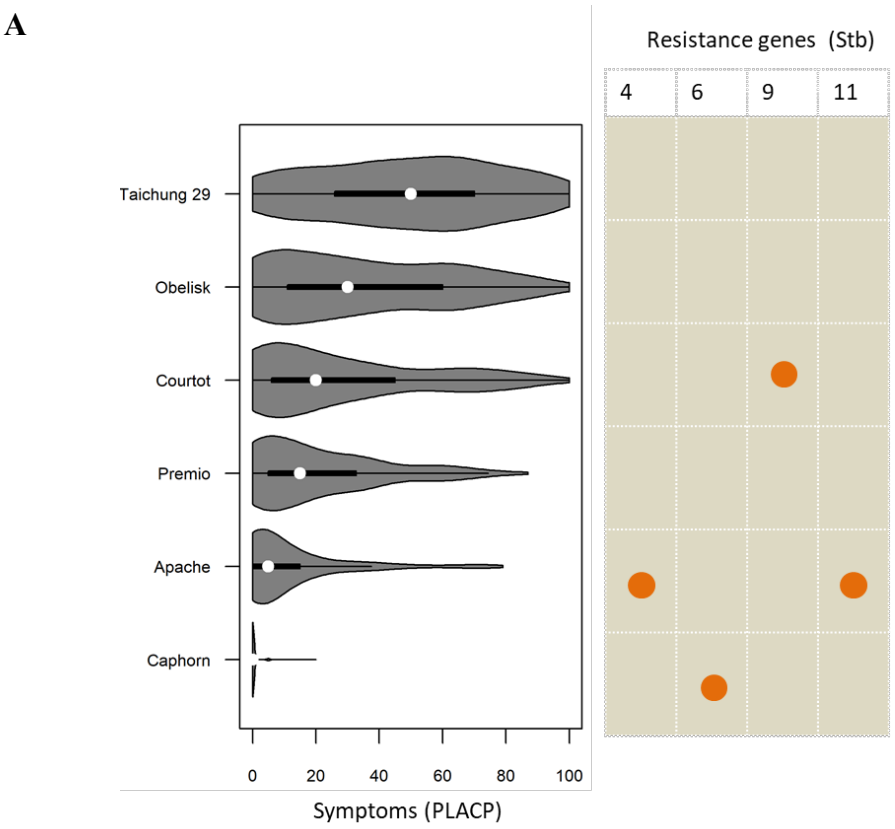


Figure 2 - Overall disease symptom distribution for the six bread wheat cultivars

A. Overall distribution across all fungal genotypes (2 ancestors and 18 evolved lineages). **B.** Orange dots indicate the presence of known resistance genes (Stb genes) in the genome of each wheat cultivar (Taichung29, Obelisk, Courtot, Premio, Apache, Caphorn).

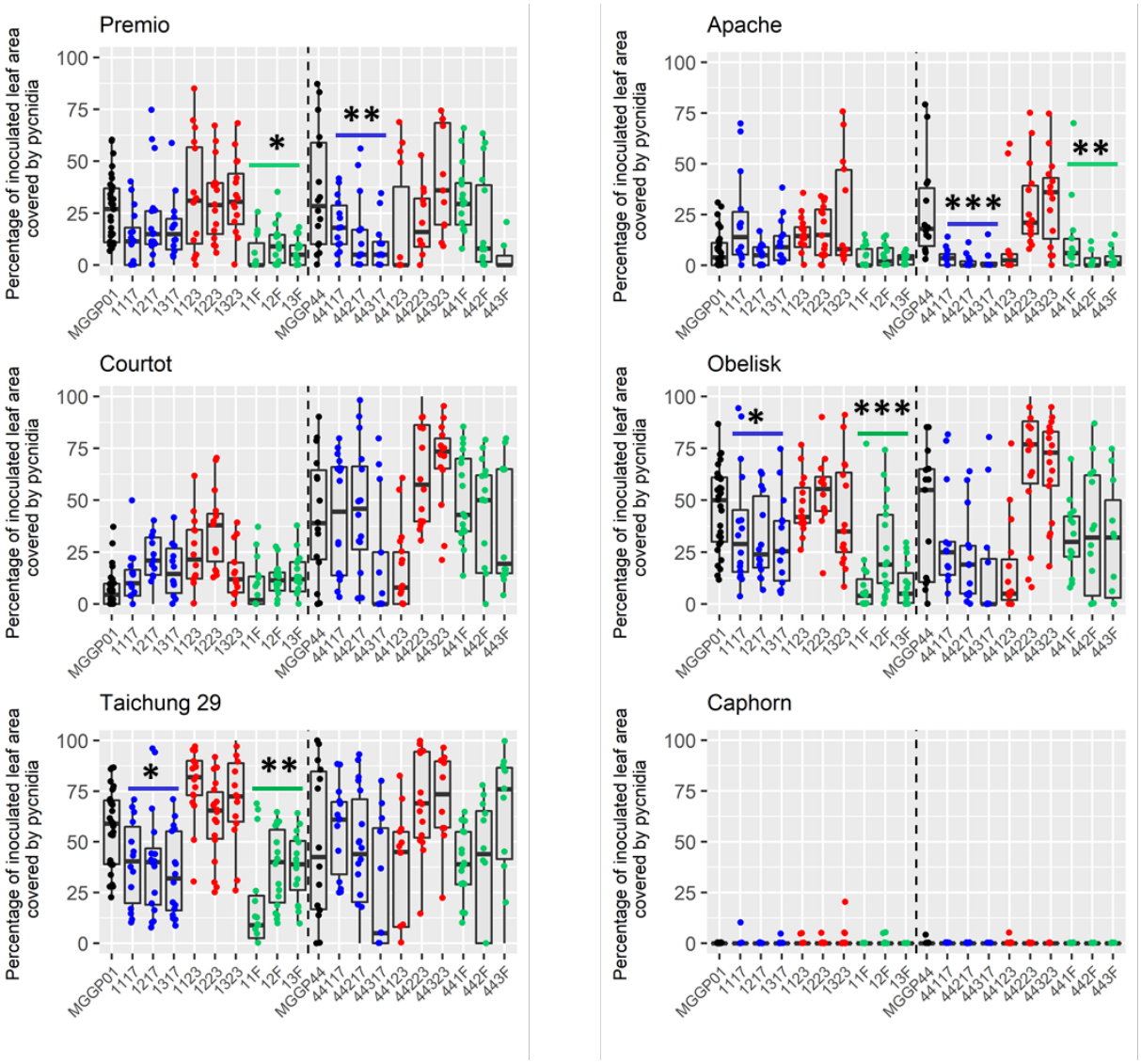


Figure 3 - Symptoms for each wheat cultivar-fungal genotype interaction

Boxplots of the distribution of the percentage of leaf area covered with pycnidia (PLACP) is shown for each wheat-fungus interaction. Selection regimes are indicated by color: ancestors (black); Stable 17°C (blue); Stable 23°C (red); fluctuating (green). Significance level of symptom changes in evolved lineages compared to their ancestor are indicated: * ($p<0.05$); ** ($p<0.1$), *** ($p<0.01$). The vertical dashed line separates lineages of MGGP01 genetic background (*left*) and MGGP44 genetic background (*right*).

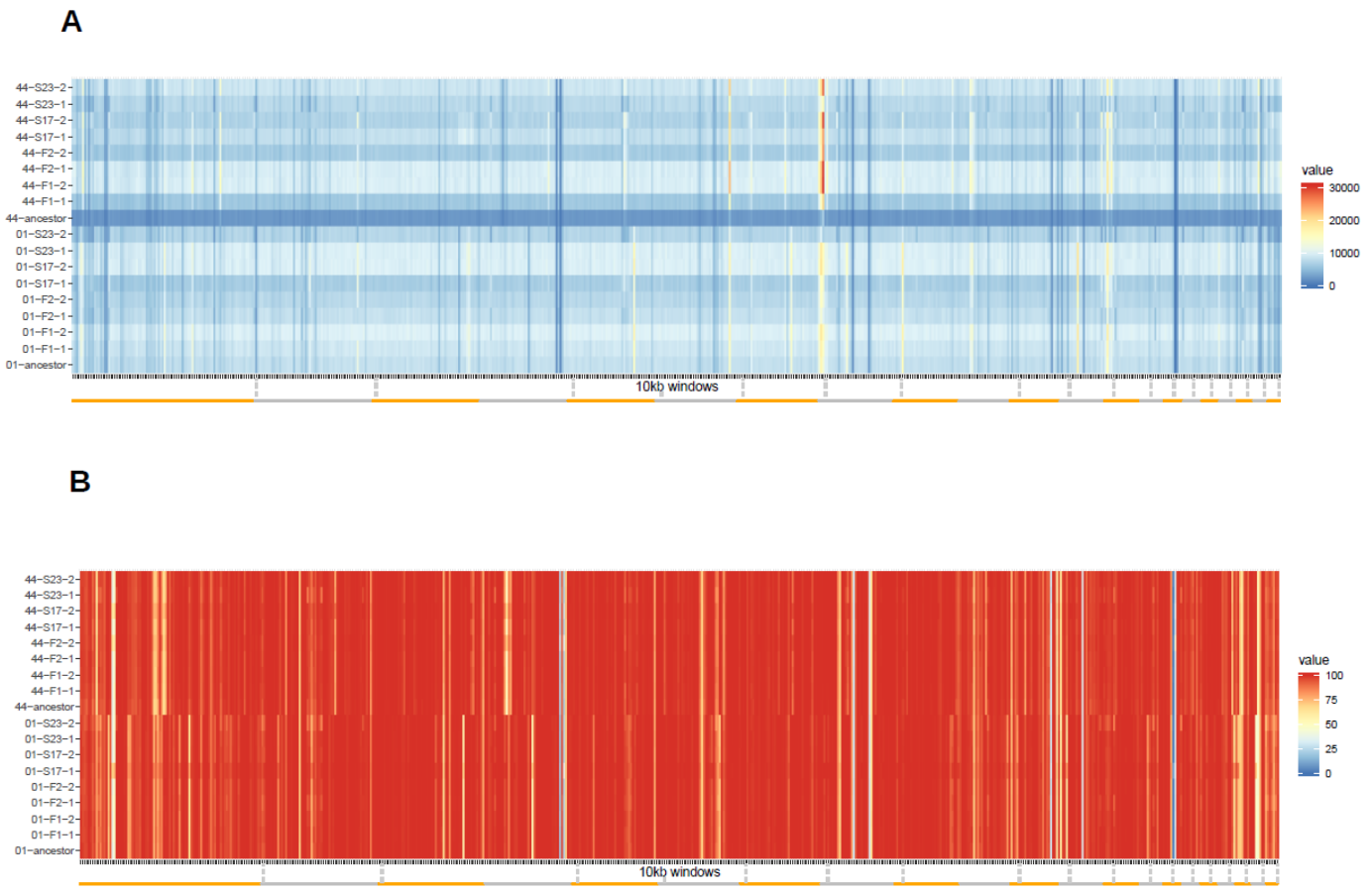


Figure 4 - Whole genome sequencing confirms the presence of 21 chromosomes for all lineages

Coverage heatmaps for all samples showing most of coverage differences are between the two ancestral strains MGGP01 and MGGP44 and the 8 evolved lineages grown at 17°C and 23°C. **A.** Average coverage along the genome (10kb windows) **B.** Percentage of very low coverage (<2X) along the genome (10kb windows). Orange and grey bars under each graph are delimiting the 21 chromosomes.

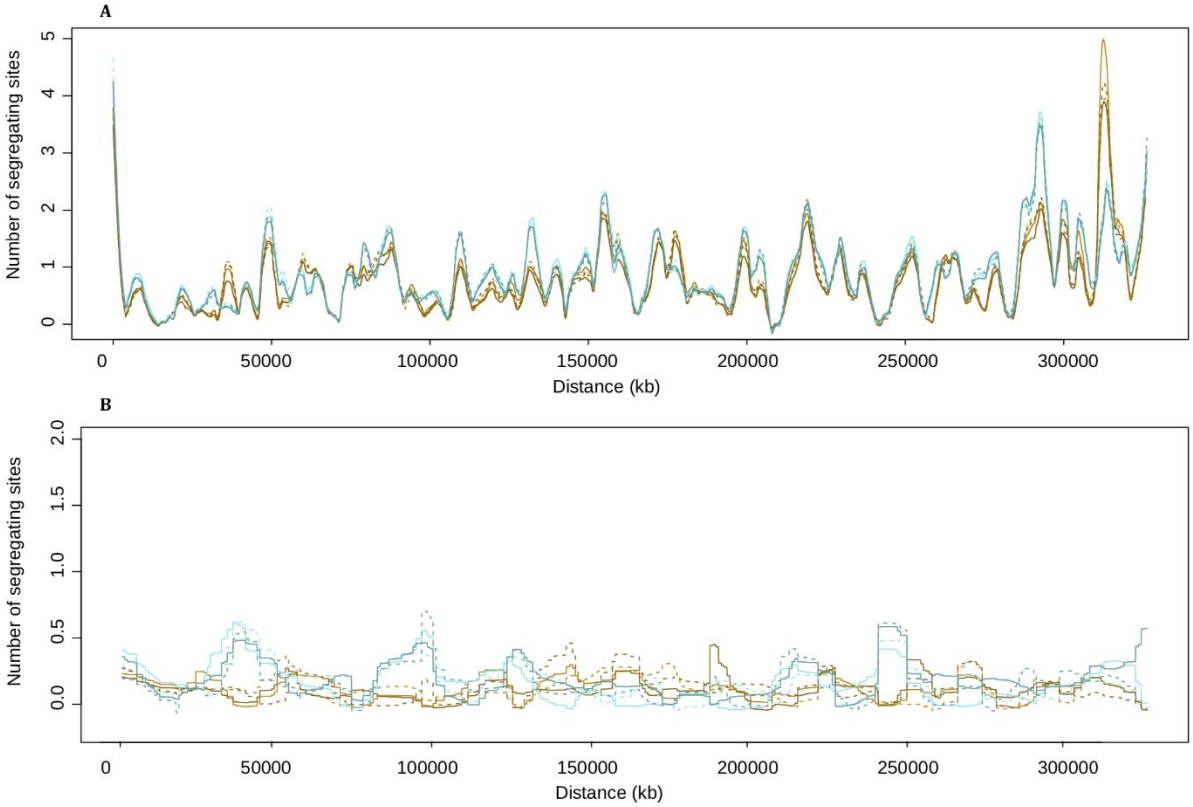
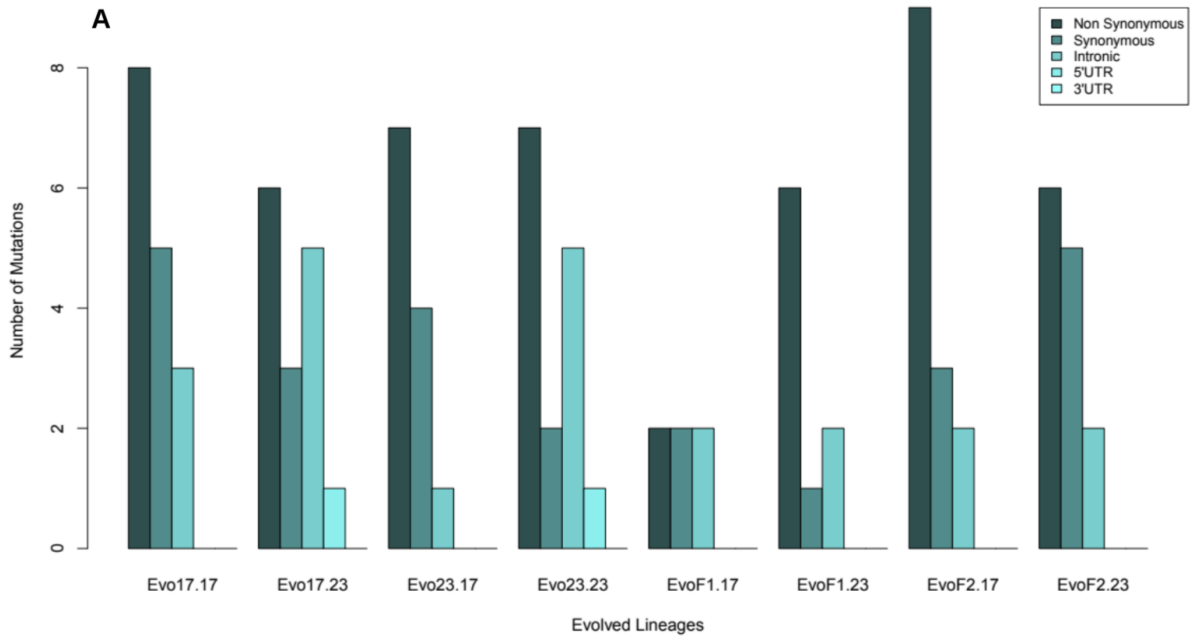
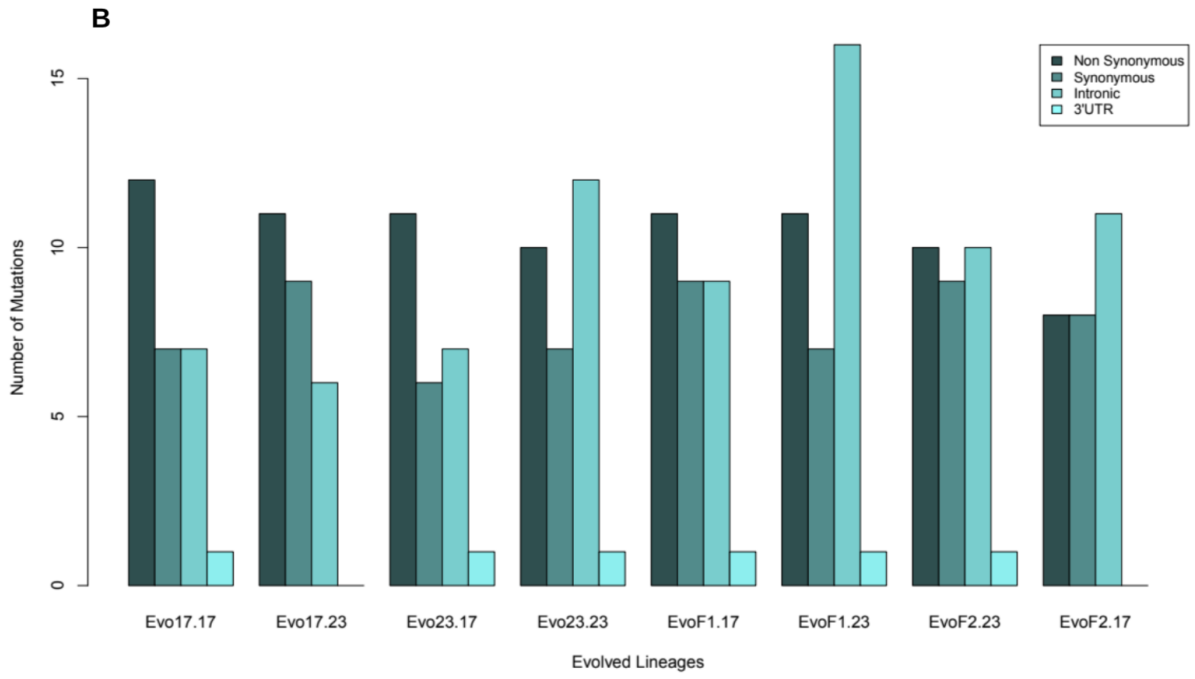


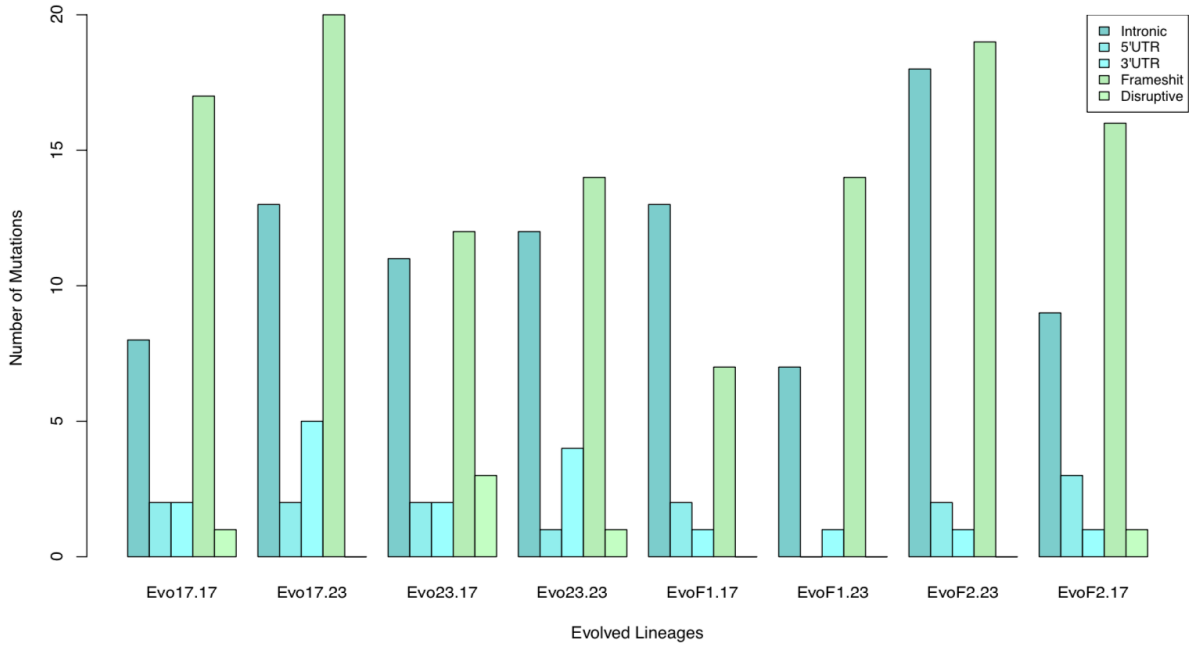
Figure 5 - Number of variable SNPs and short insertion deletions in 10kb sliding windows, with an overlap of 100bp

In total 8 groups of samples are represented: in brown, dashed brown, dark brown and dashed dark brown are for Stable 17°C + ancestor, Stable23°C + ancestor, Fluctuating-1+ancestor and Fluctuating-2 + ancestor, for background MGGP01; the same palette but blue was used for the lineages from the genetic background MGGP44. Note that the presence of the ancestor in each group allows us to identify SNPs that could be monomorphic among all evolved lineages but different from their ancestors. **A**: SNPs ; **B**: short indels.


A
Non Synonymous
Synonymous
Intronic
5'UTR
3'UTR
Number of Mutations
0
2
4
6
8
Evo17.17
Evo17.23
Evo23.17
Evo23.23
EvoF1.17
EvoF1.23
EvoF2.17
EvoF2.23
Evolved Lineages


B
Non Synonymous
Synonymous
Intronic
3'UTR
Number of Mutations
0
5
10
15
Evo17.17
Evo17.23
Evo23.17
Evo23.23
EvoF1.17
EvoF1.23
EvoF2.23
EvoF2.17
Evolved Lineages

C


Number of Mutations
0
5
10
15
20
Intronic
5'UTR
3'UTR
Frameshit
Disruptive
Evo17.17
Evo17.23
Evo23.17
Evo23.23
EvoF1.17
EvoF1.23
EvoF2.23
EvoF2.17
Evolved Lineages

D

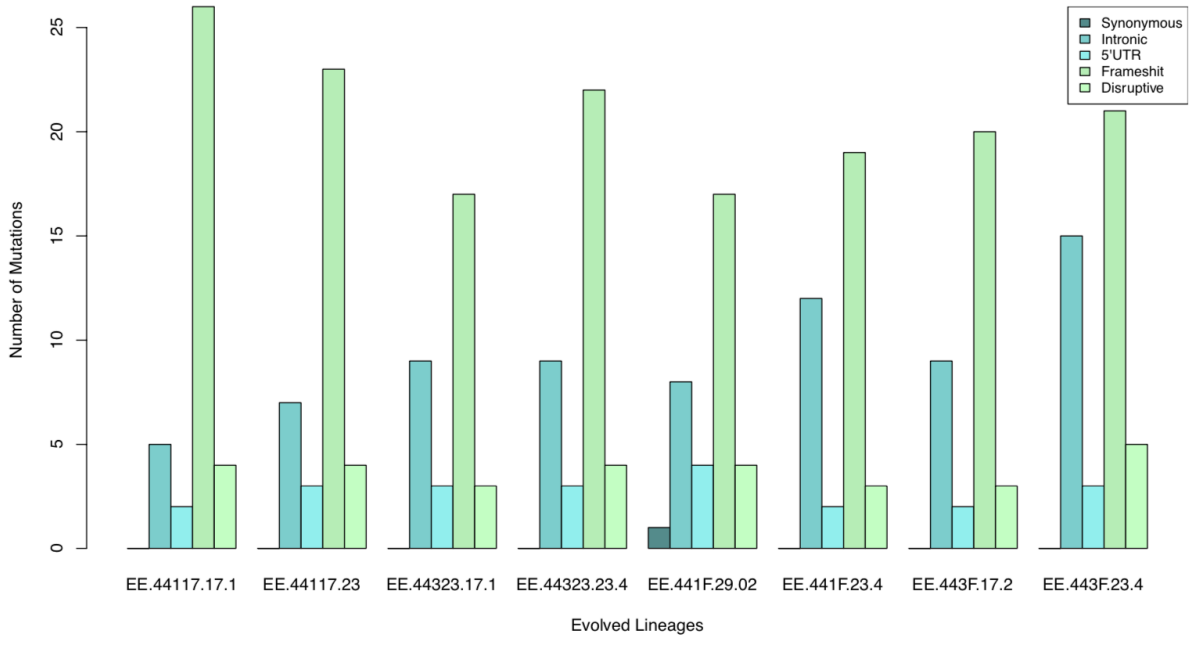

Number of Mutations
0
5
10
15
20
25
Synonymous
Intronic
5'UTR
Frameshit
Disruptive
EE.44117.17.1
EE.44117.23
EE.44323.17.1
EE.44323.23.4
EE.441F.29.02
EE.441F.23.4
EE.443F.17.2
EE.443F.23.4
Evolved Lineages

Figure 6 - Number of mutations in genes annotated in Z. tritici secretome for the genetic background MGGP01 and MGGP44

Categories of variant annotation were identified using snpSift (Cingolani et al., 2012) **A**: SNPs for MGGP01 **B**: SNPs for MGGP44; **C**: indels for MGGP01; **D**: indels for MGGP44. The location and functional consequences of the mutations are color-coded (see Figure legends).

	23°C vs 17°C		Fluctuations vs 23°C		Fluctuations vs 17°C	
	MGGP01	*MGGP44*	*MGGP01*	*MGGP44*	*MGGP01*	*MGGP44*
Premio	**3.9 x 10-4**	**0.027**	**4.7 x 10-10**	0.49	**0.021**	0.11
Apache	0.10	**2.2 x 10-10**	**3.2 x 10-5**	**8.1 x 10-7**	**0.018**	0.27
Courtot	0.39	0.090	**0.038**	0.65	0.44	0.39
Obelisk	**6.0 x 10-3**	**1.2 x 10-6**	**3.3 x 10-10**	**8.8 x 10-4**	**7.1 x 10-4**	0.17
Taichung	**2.2 x 10-9**	**6.0 x 10-3**	**3.0 x 10-13**	**0.021**	0.37	0.64

Table 1 - Comparisons of symptom development between the lineages exposed to different selection regimes

Three selection regimes were used during the experimental evolution: Stable 17°C, Stable 23°C and fluctuating temperature. All comparisons for each genetic background are presented and were tested independently for each wheat cultivars (Premio, Apache, Courtot, Obelisk, Taichung29). FDR adjusted p-values below the 5% threshold are indicated in bold.

MGGP01				MGGP44			
17°C	23°C	F-1	F-2	17°C	23°C	F-1	F-2
26011	25923	23617	25420	29820	29974	28983	29319

Table 2 - Total number of small scale mutations identified per evolved lineage