Two Is Better Than One: Studying *Ustilago bromivora*–*Brachypodium* Compatibility by Using a Hybrid Pathogen

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Pathogenic fungi can have devastating effects on agriculture and health. One potential challenge in dealing with pathogens is the possibility of a host jump (i.e., when a pathogen infects a new host species). This can lead to the emergence of new diseases or complicate the management of existing threats. We studied host specificity by using a hybrid fungus formed by mating two closely related fungi: *Ustilago bromivora*, which normally infects *Brachypodium* spp., and *U. hordei*, which normally infects barley. Although *U. hordei* was unable to infect *Brachypodium* spp., the hybrid could. These hybrids also displayed the same mating-type bias that had been observed in *U. bromivora* and provide evidence of a dominant spore-killer-like system on the sex chromosome of *U. bromivora*. By analyzing the genomic composition of 109 hybrid strains, backcrossed with *U. hordei* over four generations, we identified three regions associated with infection on *Brachypodium* spp. and 75 potential virulence candidates. The most strongly associated region was located on chromosome 8, where seven genes encoding predicted secreted proteins were identified. The fact that we identified several regions relevant for pathogenicity on *Brachypodium* spp. but that none were essential suggests that host specificity, in the case of *U. bromivora*, is a multifactorial trait which can be achieved through different subsets of virulence factors.

Keywords: fungus–plant interactions, genomics, metabolomics, proteomics

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Pathogenic fungi have a large impact on our ecosystem, infecting an array of hosts with effects on biodiversity, medicine, and agriculture. The consequences of a pathogen outbreak can be disastrous. A recent estimate based on climate modeling and the 2016 outbreak of wheat blast, caused by *Magnaporthe oryzae*, found that 7 million ha in South Asia are vulnerable to an outbreak, with a potential crop loss of 1.77 million tons (Mottaleb et al. 2018).

Knowing the host range of a fungus is important for understanding, predicting, and combatting fungal outbreaks, especially because a pathogen could take refuge in wild species, complicating disease management; for example, *Phakopsora pachyrhizi* is suspected to winter in kudzu vine between soybean seasons (Jarvie 2010). Although some fungi have narrow host ranges of only one or two plant species, others can infect more broadly; *Botrytis cinerea* is notable for being able to infect over 200 plant species (Dean et al. 2012).

It is important to bear in mind that differences in the number of hosts can reflect differences in the pathogen’s life cycle. Although *B. cinerea* is a necrotrophic fungal pathogen which kills its host to extract nutrients, other pathogens such as *Ustilago maydis* are highly specialized biotrophic pathogens and require a living host in order to complete their life cycle. In order to accomplish this, *U. maydis* carefully controls gene expression and protein secretion, both temporally (Lanver et al. 2018) and spatially (Matei et al. 2018; Schilling et al. 2014; Skibbe et al. 2010). Due to the complex regulation of gene expression required by biotrophic pathogens, they are generally expected to have a limited host range (Freeman and Beattie 2008), and researchers have cautioned that broad host ranges may be indicative of species complexes (Kruse et al. 2018). Occasionally, a biotrophic fungus is able to achieve some growth in a nonhost but is unable to complete its life cycle (Bettgenhaeuser et al. 2018; Frantzeskakis et al. 2017).

Infection of a plant by a pathogen depends on three major factors: the genotype of the pathogen (which effectors, enzymes, detectable signatures, and so on that it expresses), the genotype of the plant (the structure of its cell walls, which receptors are expressed, and more), and the environmental conditions (weather, nutrients, microbiome, and so on) that can have either a positive or negative impact on infection rates.

From the pathogen side, host–pathogen interactions are controlled by effectors; small, secreted molecules which manipulate the host’s defenses (de Jonge et al. 2011; Uhse and

Djamei 2018). Plants have evolved the ability to recognize some effectors, or their activity, and elicit a strong, specific defense response, known as effector-triggered immunity. Loss of recognized effectors has been described as underlying a host jump of *M. oryzae* to common wheat (Inoue et al. 2017) and is a potential explanation for the gene loss observed in the smut fungus *Melanopsichium pennsylvanicum* after a host jump from a monocotyledonous host to the dicotyledonous genus *Persicaria* (Sharma et al. 2014).

The ability of a pathogen to jump to a new, previously resistant host can present a serious problem because it cannot be anticipated and could result in a threat from an endemic but previously nonpathogenic microbe. This can be mediated by environmental effects such as a change in climate that creates conditions favorable for infections (Casadevall 2018). In Kentucky, an endemic strain of *Magnaporthe oryzae*, which usually infects annual ryegrass, was observed to infect wheat under the right conditions (Farman et al. 2017). Infection of new hosts can also be facilitated by interactions with other species. Although *Phytophthora infestans* cannot usually infect *Arabidopsis thaliana*, infection is possible if the plant is already colonized by *Albugo laibachii* (Belhaj et al. 2017). These rare infections can be very important because, once host resistance is overcome, it opens up a new niche for the pathogen to adapt to.

Different species which come in contact with one another may also be able to facilitate a host jump through hybridization (Brasier 2000; Olson and Stenlid 2002). The fungal pathogen *Blumeria graminis*, the causal agent of powdery mildew, occurs in host-specific varieties; *B. graminis* f. sp. *tritici* infects bread wheat while *B. graminis* f. sp. *secalis* infects rye. Triticale, the hybrid of wheat and rye, appeared to be immune to powdery mildew since its formation approximately 50 years ago. However, in 2001, triticale was reported to show signs of powdery mildew infection and it was recently found that powdery mildew in triticale is caused by *B. graminis* f. sp. *triticales*, a hybrid of *B. graminis* f. sp. *tritici* and *B. graminis* f. sp. *secalis* (Menardo et al. 2015). This example is not merely an isolated event. Pathogenic hybrids have also been described for *Verticillium longisporum*, which has arisen multiple times and displays an expanded host range compared with its parental species (Inderbitzin et al. 2011), and *Melampsora* × *columbiana*, which has an intermediate phenotype compared with its parental species and infects a greater range of poplar hosts (Newcombe et al. 2000). Many other examples exist (Depotter et al. 2016), all highlighting the need for more research on hybridization and host jumps.

We recently described a new system for studying biotrophic plant–pathogen interactions using the fungus *U. bromivora* and grasses from the genus *Brachypodium* (Rabe et al. 2016). We performed a genomic comparison between *U. bromivora* and several other smut fungi which showed that the *Brachypodium*-infecting *U. bromivora* is very closely related to the barley-infecting *U. hordei*. *U. bromivora* and *U. hordei* share 6,180 orthologs with each other and have 549 and 632 species-specific genes, respectively. Both *U. bromivora* and *U. hordei* are biotrophs capable of being cultured as haploid saprophytes but which require a plant host to complete their sexual life cycle.

In order to successfully infect a plant host, it is necessary for haploid cells of opposite mating types to mate and form a dikaryon. Mating is accompanied by a morphological switch from yeast-like budding to filamentous growth upon formation of mating hyphae. We observed that the mating locus on chromosome 1 of *U. bromivora* (Rabe et al. 2016) is very similar to that of *U. hordei* (Bakkeren and Kronstad 1994). Both fungi have a bipolar mating system with an *a* mating locus,

containing a pheromone/receptor pair necessary for mating, and a *b* mating locus, containing a homeodomain transcription factor necessary for pathogenesis, on a single chromosome forming the MAT locus. Furthermore, many fungi in the family Ustilaginaceae are known to be able to form interspecific crosses (Huang and Nielsen 1984; Kellner et al. 2011) or can be manipulated into forming interspecific crosses (Bakkeren and Kronstad 1996).

In this article, we describe *U. bromivora* and *U. hordei* hybrids which were able to form spores after infecting various *Brachypodium* spp. In order to understand the difference in host specificity between the two genetically similar fungi, we backcrossed the *U. bromivora* × *U. hordei* hybrid with *U. hordei* over several generations. Because spores can only be produced in planta, there was a constant selection for the ability to infect *Brachypodium* spp. while simultaneously diluting the *U. bromivora* genetic information which was not required for infection. This revealed three genomic loci which were linked with virulence on *U. bromivora* chromosomes 8, 13, and 22.

RESULTS

U. bromivora and *U. hordei* form viable hybrids.

We mated *U. bromivora* and *U. hordei*, showing that there is no incompatibility between the two fungi (Supplementary Fig. S2) and that the hybrid fungus is able to successfully infect *Brachypodium distachyon* ABR4 (Figs. 1 and 2) as well as the allotetraploid *B. hybridum* Bd28 (Supplementary Fig. S3). Although the overall infection timeline and symptoms of the hybrid were similar to that of wild-type *U. bromivora* (attempted *U. hordei* infections produced no symptoms on *Brachypodium* spp.), there were a few subtle differences. We noticed that the hybrid infections tended to distort the plant spikelets more than wild-type *U. bromivora* (Fig. 1D to F). In addition, the hybrid sometimes produced spores in the nodes of the *B. distachyon* ABR4 stem (Fig. 1G to I). Microscopically, we saw obvious differences neither in how the fungi grow inside the plants (Fig. 2A to C; Supplementary Fig. S4) nor between the morphology and size of the spores of the hybrid and *U. bromivora* (Fig. 2E and F).

Using the PhenoBox automated phenotyping system (Czedik-Eysenberg et al. 2018), it was possible to distinguish *U. bromivora*-infected and hybrid-infected plants from uninfected plants (Fig. 3A). Plants that were treated with either *U. bromivora* or the hybrid but did not develop symptoms were indistinguishable from uninfected control plants. The data showed that, even a few days after vernalization, the difference in plant height between control and infected plants was significant (Fig. 3B). This suggests that “failed infections” represent a complete failure to enter the plant and not merely a failure to develop spores, because one would expect colonization, with or without spore formation, to result in the same growth phenotype of the plant. An interesting phenomenon shown in Figure 3B was that *B. distachyon* ABR4 infected with the hybrid fungus failed to bolt, resulting in very little increase in height from 28 days after vernalization when compared with *B. distachyon* ABR4 infected with *U. bromivora*.

The hybrid fungus is a dikaryon; thus, it contains the complete genomic information of both *U. bromivora* and *U. hordei*. Because the hybrid was capable of infecting *B. distachyon* ABR4, it suggested either that *B. distachyon* ABR4 cannot recognize *U. hordei* or that some factor in *U. bromivora* is able to prevent the recognition. Altogether, this suggested that there are specific factors encoded by *U. bromivora* that allow it to infect *Brachypodium* spp. while the closely related *U. hordei* cannot.

Backcrossing hybrids with *U. hordei*.

In order to save time, we continued working with *B. hybridum* Bd28 for the hybrid infections because this host does not require vernalization for floral induction and is equally well infected by the fungus. We designed an experimental approach to identify the important pathogenicity regions of *U. bromivora* by repeatedly backcrossing the hybrid lines with *U. hordei* (Fig. 4). Single-spore cultures were obtained from plants infected by the original hybrid. The sporidia released

after spore germination are haploid and consist of a mixture of genomic information from *U. bromivora* and *U. hordei*. Some sporidia can be expected to have maintained the relevant pathogenicity regions from *U. bromivora* while others will have randomly lost it. These F1, hybrid-derived strains are then backcrossed with *U. hordei*. Because *U. bromivora* and *U. hordei* are extremely close genetically, we assumed that the *U. hordei* orthologs would be able to complement the missing regions of *U. bromivora*, the exception being those

Fig. 1. Macroscopic infection phenotype on *Brachypodium distachyon* ABR4. Macroscopic phenotype of ABR4 plants when uninfected or infected by either *Ustilago bromivora* or the *U. bromivora* × *U. hordei* hybrid. **A, B,** and **C,** Overall appearance of the plant at 42 days after vernalization (DAV). Scale bar represents 2 cm. **D, E,** and **F,** Close-up of the spikelets. Scale bar represents 20 mm. **G, H,** and **I,** Stem nodes. On rare occasions, the hybrid produces spores in the nodes. Scale bar represents 1 mm.

U. bromivora-specific regions important for pathogenicity in *Brachypodium* spp. After mating, further spore production was obtained by infection of *B. hybridum* Bd28. This is what maintains the selection for those regions of *U. bromivora* that are necessary for infection. Following several rounds of backcrossing, we obtained a population of hybrid strains of various generations with a decreasing proportion of their genome coming from *U. bromivora*, some of which were still able to infect *B. hybridum* Bd28 and others which had lost this ability.

We observed a clear decrease in the percentage of strains that were able to cause infection symptoms in each subsequent generation (Fig. 5A). In addition, although the original hybrid of *U. bromivora* × *U. hordei* led to the development of infection symptoms on a median of 60% of infected plants, we saw a rapid drop in infection strength as we progressed through the generations (Fig. 5B). This showed that the loss of *U. bromivora* genomic regions had a negative, quantitative effect on the ability of the hybrids to infect *B. hybridum* Bd28.

We analyzed the genomic sequences of 109 hybrid strains by next-generation sequencing (NGS) in order to identify which regions of the genome were contributed from *U. bromivora* and to correlate that information with which strains were able to cause symptom development on *B. hybridum* Bd28 (Supplementary Table S2). By aligning the Illumina reads to perfectly match *U. hordei* genes, we could count those reads that were either derived from *U. hordei* or perfectly conserved. We could then take the remaining reads and map them onto the

U. bromivora genes. This allowed us to see which genes were present (defined as at least five reads, covering 90% of the gene length) and express that as a percentage of the total number of *U. bromivora* genes or the number of genes which are either shared or *U. hordei* specific. This provided a simple proxy for assessing how the genomic contribution changes through the generations and showed that the amount of the genome coming from *U. bromivora* decreases through the generations and the amount contributed from or shared with *U. hordei* increases (Fig. 5C). The percentage of *U. bromivora* gene content in the hybrid strains was correlated with their ability to cause infection symptoms on plants ($r = 0.407988$).

Finally, when crossing *U. bromivora* MAT1 with *U. hordei* MAT2, we saw a mating-type bias where 320 of 342 sporidia (93.56%) from 99 spores were MAT1. Although there was variation in later generations as to whether MAT1 or MAT2 predominated, there was always a strong bias to a specific mating type. This indicates that the MAT1 bias that we previously reported for *U. bromivora* is based on a dominant in trans acting factor that is genetically linked to the region close to the *mat1* locus on the fungal sex chromosome (Rabe et al. 2016).

Genomic regions responsible for host specificity.

The 109 Illumina-sequenced hybrids were divided into 31 strains which were observed to cause infection symptoms on *B. distachyon* Bd28 and 78 strains which were not. Combining

Fig. 2. Microscopic infection phenotype on *Brachypodium distachyon* ABR4. Microscopic phenotype of ABR4 plants when uninfected or infected by either *Ustilago bromivora* or the *U. bromivora* × *U. hordei* hybrid. **A, B, and C,** Maximum intensity z-stack projection showing fungal and plant cells in the stem, 3 weeks after vernalization. Plant cell walls are stained with propidium iodide and fungal cells are stained with wheat germ agglutinin-Alexafluor. Scale bar represents 50 μ m. **D, E, and F,** Differential interference contrast microscopy images of the spores released from grinding the spikelet. The uninfected spikelet shows no spores at all whereas both *U. bromivora*- and hybrid-infected spikelets stain the water black with the spores. Scale bar represents 10 μ m.

the phenotypic and genotypic data allowed us to generate associations between them. We used two independent methods to identify regions and genes that were required for pathogenicity on *B. hybridum* Bd28.

In our first approach, we looked exclusively at nonsynonymous changes in the hybrids compared with both the *U. hordei* or *U. bromivora* reference genomes. This allowed us to find genes which may have accumulated mutations during passing and mating and identify whether those mutations affected virulence. In total, 17,231 nonsynonymous changes were identified, 5,214 of which were more common in the infecting hybrid strains, with 51 reaching statistical significance ($P < 0.01$) (Fig. 6A). This gives a list of 35 genes which are associated with pathogenicity (Supplementary Table S3) but none of which are predicted to be secreted and, thus, would not likely fulfill the function of an effector. Nonsynonymous mutations found between hybrid strains and the *U. hordei* genome are not expected to have an effect on the ability of the hybrid to infect. Although it is possible that mutations in *U. hordei* genes may have some effect on infection, given the lack of predicted secreted proteins for both *U. hordei* and *U. bromivora*, it is more likely that these mutations lead to a general improvement in growth which then contributes to an improved outcome in planta.

An additional 11,978 nonsynonymous changes were associated with those strains which could no longer infect, with 1,179 reaching statistical significance ($P < 0.01$) (Fig. 6B). The 198 genes represented by these single-nucleotide polymorphisms (SNPs) (Supplementary Table S4) are primarily found in *U. bromivora*, with fairly even representation across the genome. Only four are predicted to be secreted; two from chromosome 1 are predicted to have a membrane function, one from chromosome 3 is predicted to be involved in intracellular protein transport, and the last, on chromosome 8, has no functional annotation but was identified as an ortholog of

U. maydis mig1. Functional annotation suggests that the majority of these 198 genes which have gene ontology (GO) terms are involved in DNA binding and endonuclease activity and located in either cell membranes or the mitochondria. It seems likely that most of the affected genes are important for the growth of the hybrids and mutations result in impaired infection even when the necessary effectors are present. Given the large difference in the occurrence of these SNPs between *U. bromivora* and *U. hordei*, it could also be suspected that these are genes which are not easily compensated by the *U. hordei* genome. Any impaired function from the *U. hordei* SNPs would be compensated for during infection by the full genome of *U. hordei* during backcrossing. For *U. bromivora*, there is no such opportunity to compensate for species-specific pathways or structural changes that have taken place over evolution. The few *U. hordei* SNPs that are associated with a reduction in pathogenicity are then likely either dose dependent or represent dominant, gain-of-function mutations which can have wide-ranging effects.

In our second approach, by using a combination of multiplexed shotgun genotyping (MSG) (Andolfatto et al. 2011) to determine ancestry-specific SNPs (SNPs which identify the sequence as originating from either *U. bromivora* or *U. hordei*) and the R package r/ql (Broman et al. 2003; R Core Team 2013) to analyze the resultant files, it was possible to generate associations with pathogenicity over the entire *U. bromivora* genome. The outputs of the analysis not only showed the strength of association between SNPs and pathogenicity (Fig. 7) but also allowed us to predict the parental contribution of each chromosome (Supplementary Fig. S5).

We assessed the association of 260,109 ancestry-specific SNPs with two different phenotypes: a binary trait considering whether a strain was capable of infecting *B. hybridum* Bd28 and a quantitative trait based on the percentage of infected plants which displayed symptoms. Three regions, occurring on

Fig. 3. Phenotypic differences between uninfected, *Ustilago bromivora*-infected, and hybrid-infected *Brachypodium distachyon* ABR4. Although the end phenotypes of the *U. bromivora* × *U. hordei* hybrid and *U. bromivora* infection are quite similar, there are subtle differences in their effects on the plant. **A**, Principal components analysis (PCA) of uninfected (pale yellow), *U. bromivora*-infected (red), and hybrid-infected (blue) *B. distachyon* ABR4 plants 42 days after vernalization based on visual data collected by the PhenoBox automated phenotyping system. Lighter colored spots are plants which were infected with *U. bromivora* or the hybrid but did not develop symptoms. These failed infections are indistinguishable from uninfected plants on the PCA. *U. bromivora*- and hybrid-infected plants are both distinct from the uninfected plants and from each other. The parameters describing the loading of the displayed principal components (PCs) can be seen in Supplementary Table S1. Further details on the parameters determined by the PhenoBox are available in Czedik-Eysenberg et al. (2018). **B**, A more detailed look at a single phenotypic trait (plant height) when measured over time. *U. bromivora* and the hybrid are both significantly smaller than uninfected plants. From 28 days after vernalization, there is a clear difference between hybrid-infected and *U. bromivora*-infected plants because the former fails to undergo bolting. Error bars indicate standard deviation. P values were corrected for multiple testing using the Benjamini-Hochberg method; *, **, ***, and **** indicate $P < 0.05$, 0.01, 0.001, and 0.0001, respectively.

U. bromivora chromosomes 8, 13, and 22, showed a significant association with the ability to infect at an α value of 0.01. Only the region on chromosome 8 was associated with both the binary and the quantitative measure of infection. An association with chromosome 13 was found for the quantitative trait and with chromosome 22 for the binary trait. If we lowered the threshold to an α value of 0.05, then those three regions are the only ones which reach significance for both traits. Despite its prominence in the association tests, the region on chromosome 8 is not essential for virulence because several strains maintain the ability to infect while lacking part or all of the *U. bromivora* sequence. This indicates that host specificity in *U. bromivora* is a multifactorial trait which is influenced by at least three loci and that infection can be achieved through multiple, independent combinations of genes.

Altogether, we identified 75 genes in regions associated with virulence (Supplementary Table S5). Of these, six lack an ortholog in *U. hordei* and all occur on chromosome 8. Because we were looking for factors unique to *U. bromivora* when compared with *U. hordei*, these may be of special significance. One of the genes, *UBRO_04981*, is identified as a probable GFA1-glucosamine fructose-6-phosphate transaminase and can be

annotated with GO terms relating to chitin biosynthesis and carbohydrate binding. Although not identified as secreted, this does identify a function where a mutation could potentially improve pathogenesis because recognition of chitin can lead to pathogen-associated molecular pattern-triggered immunity. Four of the other five genes (*UBRO_20681* to *UBRO_20684*) occur sequentially on chromosome 8. This is interesting because it might suggest that these genes have a similar origin or are clustered together for a shared function. Two have DNA-related functions while the other two have no functional annotation at all.

Of the 75 potential virulence genes, 9 are predicted to be secreted, although three (*UBRO_04936*, *UBRO_06823*, and *UBRO_06828*) are likely to have enzymatic functions unrelated to virulence. Furthermore, the *U. maydis* orthologs of *UBRO_04936* and *UBRO_06828* are downregulated during infection (Lanver et al. 2018). *UBRO_06823* has two *U. maydis* orthologs, one of which is upregulated during late infection and one of which is upregulated at a low level during infection. *UBRO_06823* has a function in glucan catabolism, which could serve to help weaken the plant cell wall but which may just be involved in harvesting sugar from the surrounding environment.

Of the 75 virulence candidates, 2 genes (*UBRO_04990* and *UBRO_04923*) are related to *mig1*, which is known to be upregulated by *U. maydis* in planta and is likely an effector (Basse et al. 2000). The *U. maydis* ortholog of *UBRO_04923* is *UMAG_12126*, which has been observed to have a virulence defect upon deletion (Uhse et al. 2018). All of the genes encoding predicted secreted proteins of unknown function or those related to *mig1* are located on chromosome 8. Of the final four effector candidates (*UBRO_05003*, *UBRO_04986*, *UBRO_04984*, and *UBRO_04921*), only *UBRO_04921* does not have an ortholog in *U. maydis* and all of the orthologs are significantly upregulated during infection (Lanver et al. 2018). Most of the orthologs are upregulated approximately 10-fold during infection, with the exception of *UBRO_05003*, which has the GO annotation “defense response to other organism,” which is only upregulated approximately 5-fold.

DISCUSSION

Our study used hybridization of two closely related smut fungi to gain an understanding of host specificity. We demonstrated that *U. bromivora* and *U. hordei* are capable of forming a viable hybrid which can cause infection symptoms on *Brachypodium* spp. similar to, and perhaps even stronger than, the parental *U. bromivora*. We cannot say whether this is due to the combined complement of effector proteins or some other effect of the combined genomes.

A particularly interesting observation was that there were several instances where there were large numbers of ancestry-specific SNPs from both *U. bromivora* and *U. hordei*. When MSG tried to predict ancestry of the chromosomes, these regions remained ambiguous and we interpreted this as evidence of partial or complete duplication of chromosomes. Genome sequencing was performed on the haploid sporidia; thus, it was unexpected to see multiple copies of a chromosome or region of a chromosome. Chromosomal duplications are one of the reasons that the fraction of *U. bromivora*-specific reads and shared or *U. hordei*-specific reads do not always add up to 1. The genome content in our analysis is also affected by sequencing depth in some cases, number and distribution of SNPs in a gene, and other technical biases.

One region was particularly notable in terms of duplication: a 265-kb region of *U. bromivora*, containing the mating type locus, was present in nearly every hybrid. Even when there was evidence of a complete *U. hordei* version of chromosome 1, we also saw *U. bromivora*-specific SNPs at the center of

Fig. 4. Schematic of the hybrid backcrossing. Schematic describing the hybrid backcrossing approach. Running down the left-hand side is the name of each hybrid generation. On the lighter background, haploid parental strains (generation [Gen] P) each contain 100% of their respective genome. These parental strains are mated to form an infectious dikaryon with 100% of each parent's genome (generation dP, where the “d” indicates the dikaryon formed by mating with *Ustilago hordei*). From generation F1 onward, only the haploid hybrid is shown in detail. Between each generation, the hybrid is mated with *U. hordei* to form a dikaryon which is used to infect *Brachypodium hybridum* Bd28. This dikaryotic form is depicted by a black dot on the line between haploid generations and both produces the spores for the next generation and shows which strains are still pathogenic. Different F1 strains were used to create several sets of independent F2 progeny. The main hybrid line, marked in bold, shows the strains descended from the same parent in each generation. At the bottom right of each set of hybrids is a pair of numbers that represents the number of strains which were able to cause infection symptoms on plants as well as the total number of strains tested.

chromosome 1, creating ambiguity in predicting ancestry. We believe that this region encodes a spore-killer gene which results in a mating-type bias. Dominant spore-killer traits which lead to a mating-type bias have been described in several other fungi (Kathariou and Spieth 1982; Turner and Perkins 1979). Previously, we reported that *U. bromivora* has a mating-type bias where all 225 sporidia (100%) from 21 spores tested were found to be MAT1 (Rabe et al. 2016) though the cause of the bias remained speculative. This mating-type bias persisted in our hybrid crosses, although *U. hordei* does not display a mating-type bias. This provides evidence that the mating-type bias is a dominant trait carried by *U. bromivora* MAT1, although the mechanism of action is still unknown. Interestingly, likely due to the genomic rearrangements accompanying hybridization, the mating type which was favored by the bias differed between generations. However, all of the strains analyzed here maintained this spore-killer region, presumably because those which did not were unable to survive saprophytically on plate without being rescued by a strain containing it. The sole exception to this rule was F4 strain 72784, which has a reduced number of *U. bromivora*-specific SNPs in that region and differs in mating type from the other F4 strains in the analysis.

Excluding the locus on *U. bromivora* chromosome 1 and the unassigned *U. bromivora* genome scaffold, which has few SNP markers, we observed ambiguous ancestry and potential duplications in 40 to 50% of all hybrid strains (e.g., chromosome 9 in the F3 and F4 generations). Genomic changes during

hybridization have been observed before in diverse lineages, including yeasts (Pryszcz et al. 2015), plants (Gaeta et al. 2007), and marsupials (O'Neill et al. 2001). It is possible that chromosomal differences may arise to aid speciation and the rearrangements seen here could indicate partial incompatibility between *U. bromivora* and *U. hordei*. *Saccharomyces* spp. can be divided into two groups: sensu stricto and sensu lato. It has been shown that interspecific hybrids between two yeasts in the sensu stricto group maintain both sets of parental chromosomes (Marinoni et al. 1999) and chromosomal stability approximately equal to a diploid parental strain (Kumaran et al. 2013). However, interspecific matings involving a sensu lato species show much lower rates of viable hybrid formation and a failure to maintain both complete sets of parental chromosomes (Marinoni et al. 1999). It is unclear exactly what effect genomic rearrangements might have on pathogenicity beyond the specific genetic composition of the hybrids but changes in gene expression, which could potentially have virulence effects, have been observed in hybrid fungal genomes (Cox et al. 2014). It would be interesting if future research could identify any changes in gene expression among the hybrids due to these rearrangements.

By associating genetic differences between the hybrids with their ability to cause infection symptoms, we identified three regions in *U. bromivora* which are likely to be important for *U. bromivora* pathogenicity on *Brachypodium* spp. These regions occur on *U. bromivora* chromosomes 8, 13, and 22. The

Fig. 5. Hybrid strain virulence and *Ustilago bromivora* genome content decreases with each generation. **A**, Percentage of hybrid strains capable of causing infection symptoms on *Brachypodium hybridum* Bd28 when mated with *U. hordei* decreases with each generation. **B**, For hybrid strains capable of causing infection symptoms, the strength of the infections decreases with each generation. The strength of the infection is measured as the number of plants infected which go on to develop symptoms for a particular hybrid strain. For the dP generation (*U. bromivora* × *U. hordei*), the individual points show independent infections. For the backcrossed generations, the individual points show specific hybrid strains. No dF4 strains were observed to cause symptoms. **C**, With each generation, the percentage of *U. bromivora* genes (red) detected by *U. bromivora*-specific reads in the hybrid strains decreased while the percentage of *U. hordei* or shared genes (blue) detected by *U. hordei*-specific or shared reads increased.

strongest association with hybrid virulence came from *U. bromivora* chromosome 8. We showed that this region has more putative effector proteins but it is not essential for virulence, which suggests that infection can be accomplished with different effector complements.

It could be possible that there was originally one major factor allowing *U. bromivora* to colonize *Brachypodium* spp. but, over time, its significance has faded during adaptation. The evidence we provide does not exclude this possibility but it doesn't provide strong support for it, either. It seems more likely that this host specificity might have arisen slowly rather than as a specific event. Perhaps the ancestral species of *U. bromivora* and *U. hordei* encountered *Brachypodium* spp. due to a migration on the plant or fungal side, or a change in climate allowed infection, similar to *M. oryzae* infection of wheat (Farman et al. 2017). With no particular adaptation to the host, the infection would be very weak and only occasionally lead to spore formation, not unlike *Puccinia striiformis* f. sp. *tritici* on *Brachypodium* spp. today (Gilbert et al. 2018). These weak infections would allow for natural selection to act on the ancestral fungus in order to adapt to better infect and reproduce on *Brachypodium* spp.

Although our work here was focused on host specificity of *U. bromivora* infecting *Brachypodium* spp., the hybrid fungus approach is not limited to this system. If it were shown that

U. bromivora alone is unable to infect barley and that the hybrid maintains the ability, then one could investigate host specificity of *U. hordei* on barley in much the same manner. Given the widespread possibilities for hybridization among members of the family Ustilaginaceae (Huang and Nielsen 1984; Kellner et al. 2011) and the possibility of enabling hybridization through manipulating mating loci (Bakkeren and Kronstad 1996), one could imagine hybrid infections as a useful tool for examining host-specific pathogenicity in smuts generally and, perhaps, even more widely.

MATERIALS AND METHODS

Strains, growth, and infection.

We used *U. bromivora* strains Mat 1 and Mat 2, which were previously described (Rabe et al. 2016), and *U. hordei* Mat 1 (strain 4875-4) and Mat 2 (strain 4875-5), which were provided by Jan Schirawski. Growth conditions for *U. bromivora*, *U. hordei*, *B. distachyon* ABR4, and *B. hybridum* Bd28 as well as the infection protocol were all previously described (Rabe et al. 2016). *U. bromivora* and the *U. bromivora* × *U. hordei* hybrids were originally used to infect *B. distachyon* ABR4. Images of whole plants and the phenotypic data for a comparison of growth effects were acquired using the PhenoBox

Fig. 6. Potentially damaging single-nucleotide polymorphisms (SNPs) associated with the ability to infect *Brachypodium hybridum* Bd28. An analysis of potentially damaging (nonsynonymous and loss-of-function) SNPs in hybrids which may be associated with the ability to infect *B. hybridum* Bd28. These SNPs were obtained by comparing the reads to the reference genomes and can be found in both parental species. They are divided into SNPs that are **A**, found more often in strains that are capable of infecting and **B**, more often in strains that are not capable of infecting. Points above the red line represent SNPs which are overrepresented in infectious samples with a P value < 0.01 . P values were corrected for multiple testing using the Benjamini-Hochberg method. Due to space constraints, not all scaffolds or chromosomes are labeled.

automated plant-phenotyping platform (Czedik-Eysenberg et al. 2018). Whole plants were stored in 100% ethanol before microscopy and images of fungal infections in plant tissue were acquired following the protocol of Dawson et al. (2015). Spores from the hybrid-infected plants were isolated from infected spikelets and germinated as previously described (Bosch and Djamei 2017). The mating type of the haploid hybrid strains was determined, they were mated with *U. hordei* of the opposite mating type (Mat 1 hybrid with Mat 2 *U. hordei* and

vice versa), and the dikaryotic fungi were used to infect *B. hybridum* Bd28. *B. hybridum* Bd28 was used because of the easier growth conditions and shorter time to flowering compared with *B. distachyon* ABR4 (Rabe et al. 2016).

DNA extraction and PCR.

Fungal cultures were grown in potato dextrose medium and genomic DNA (gDNA) was extracted using a phenol/chloroform extraction. Mating type was determined by a PCR approach

Fig. 7. Linkage of ancestry markers with the ability to infect *Brachypodium hybridum* Bd28. Certain regions of the *Ustilago bromivora* genome show an association with the ability to infect *B. hybridum* Bd28. Horizontal lines show the logarithm of odds (LOD) score cut-off of 4.13, representing $\alpha = 0.01$. The LOD score is a measure of the association of a particular single-nucleotide polymorphism with the phenotype under consideration. Regions on chromosome 8, 13, and 22 exceed the threshold. **A**, LOD scores of chromosomal regions when the phenotype is defined as a binary trait of causing infection symptoms on *B. hybridum* Bd28 or not. **B**, LOD scores of chromosomal regions when the phenotype is the percentage of total plants that show infection symptoms. Due to space constraints, the mitochondria and an unassigned scaffold are not labeled.

based on Kellner et al. (2011). Primers were designed to amplify the fungal mating loci *U. bromivora* pra1 (GCTTTGTTGAGTGT CGATGC and ACCAATGTCGCCATGTACTC, 494-bp product) and *U. hordei* pra2 (TCCCTCTCTTCTTCGTGGTT and GCTGGAGTAAGCAGAAGTC, 418-bp product). Mating could also be shown by inducing filamentation by growing a mixture of two compatible mating types on potato dextrose plates with the addition of activated charcoal (10 g/liter).

NGS.

Extracted fungal gDNA was adjusted to 2.5 ng/μl after quantification using picogreen dye (Thermo Fisher Scientific) to exclusively measure double-stranded DNA. We followed a modification of the Illumina library preparation protocol prepared by Sergey Kryazhimskiy (V. 2.1; 2013-06-06) which was later published, after further modifications, by Baym et al. (2015). Barcoding was done using an Illumina Nextera kit (Illumina) for labeling 24 samples. We performed library amplification using either Veraseq polymerase (Biozyme) or Phusion polymerase (Thermo Fisher Scientific) and size selection with AMPure XP beads (Beckman Coulter). Fragment profiles of the libraries were determined with a Fragment Analyzer (Advanced Analytical) and libraries were pooled to equal concentrations based on either the concentration determined by the Fragment Analyzer or a second round of quantification with picogreen fluorescent spectrometry. The libraries were sequenced using Illumina HiSeqV4 PE125 on a HiSeq2500 at the Vienna BioCenter Core Facilities (VBCF) NGS Unit.

To get an idea of how well the sequencing covered the *U. bromivora* part of the hybrid genome and to identify the presence or absence of specific genes, we performed the following steps. The demultiplexed .bam files received from the NGS facility were converted to paired .fastq files with the use of BAM2FQ.jar. BAM2FQ.jar is a VBCF NGS modification of the Picard tools from the Broad Institute to include a graphical user interface. These paired fastq files were imported into CLC Genomic Workbench 9 (Qiagen), aligned perfectly first to the *U. hordei* protein-coding genes and then to the *U. bromivora* protein-coding genes, and summary tables for both alignments were produced. The *U. bromivora* reference genome (GCA_900080155.1) was sequenced from *U. bromivora* UB1 (formerly strain UB2112) (Rabe et al. 2016). The *U. hordei* reference genome (GCA_000286035.1) was sequenced from *U. hordei* Mat 1 (strain Uh4857-4) (Laurie et al. 2012).

The workflow used *U. bromivora*-specific reads only to show which genes were present. Due to how closely related the two fungi are, it was not always possible to distinguish between them but regions from *U. bromivora* that were identical to those from *U. hordei* were not expected to be relevant to pathogenicity.

In order to make comparisons between different hybrid strains, the gene-by-gene information was reduced to strain-specific summaries and these were joined together into a single table through custom bash and R (R Core Team 2013) scripts.

The install file for the CLC Genomic Workbench 9 workflow and the custom bash and R scripts are archived on Zenodo.

Virulence associations with potentially damaging SNPs.

Initial quality control, adapter removal, and filtering of raw sequence reads was performed using Trim Galore! (v0.4.5; Babraham Bioinformatics) in the default mode. Processed paired-end reads were mapped against *U. hordei* and *U. bromivora* chromosomes using BWA mem (v0.7.17) (Li and Durbin 2009). Subsequently, we marked all potential PCR duplicates using Picard Tools (v2.18.3) (Broad Institute) to reduce the amount of potential technical artifacts. SNPs and

indels were called using GATK HaplotypeCaller (v3.8-1) (McKenna et al. 2010) in gVCF mode. We applied hard-filtering on raw variant calls according to 'GATK Best Practices' to remove potential false-positive variants. Due to the absence of any gold standard variant set, we used the set of remaining high-confidence variants to apply the GATK Base-Recalibrator on the PCR-marked BAM files. Afterward, the final variant calling was performed. Variant filtering was done using the same parameters as before. The resulting SNPs were annotated using snpEff (v4.3q) (Cingolani et al. 2012). Subsequently, SNPs with putative damaging effects (non-synonymous and loss-of-function) were used as input for association tests using PLINK (v1.9) (Purcell et al. 2007). Association *P* values were corrected for multiple testing according to Benjamini and Hochberg (1995).

MSG and r/qtl.

In order to estimate the likely ancestry of chromosomal regions within the hybrids, we made use of the MSG technique (Andolfatto et al. 2011) on the first of the paired fastq files. The ancestry tables which are outputted by MSG were cleaned to remove SNPs where more than 10% of the strains lacked data and were then submitted to pull_thin to remove redundant SNPs. The cleaned ancestry files were then used as input to r/qtl (Broman et al. 2003) with the read_cross function, along with information on which strains caused symptoms on plants in order to show linkage between SNPs and pathogenicity over the whole genome. We used 1,000 permutations of r/qtl's scanone function to determine that measuring association with a logarithm of odds (LOD) threshold of 4.13 would be equivalent to an α of 0.01. Genes that fell within regions with a LOD score ≥ 4.13 were considered as potential virulence candidates. For chromosome 22, the relevant region is only approximately 500 bp in length but this falls entirely within the gene *UBRO_08780*, which was then added to the potential virulence candidates list.

In order to estimate the size of the region involved in the mating-type bias, we modified the analysis above. Instead of performing the r/qtl analysis with the virulence data, we used the mating type of the hybrid strains. Due to presence of the mating-type bias region in nearly every strain, there is a sudden, strong drop in association scores over that region. By identifying which SNPs were assigned a LOD score < 0.5 , we were able to assign specific coordinates to this region. The region that was identified fell within a larger region that had previously been identified in unpublished data as important for the mating-type bias.

Functional annotation of potential virulence candidates.

Potential functions of the candidate genes were obtained through annotations of the genomes (Laurie et al. 2012; Rabe et al. 2016) and via analysis with Blast2GO (Götz et al. 2008). We obtained functional annotations in Blast2GO by integrating the built-in Interpro scan with the BLAST search, mapping, and annotation, all using the default settings.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

VBCF NGS Unit: <https://www.viennabiocenter.org/facilities/>
 Broad Institute Picard tools: <http://broadinstitute.github.io/picard>
 pull_thin software: https://github.com/dstern/pull_thin
 read_cross function: https://github.com/dstern/read_cross_msg
 Install file on Zenodo: <https://zenodo.org/record/2563198>

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