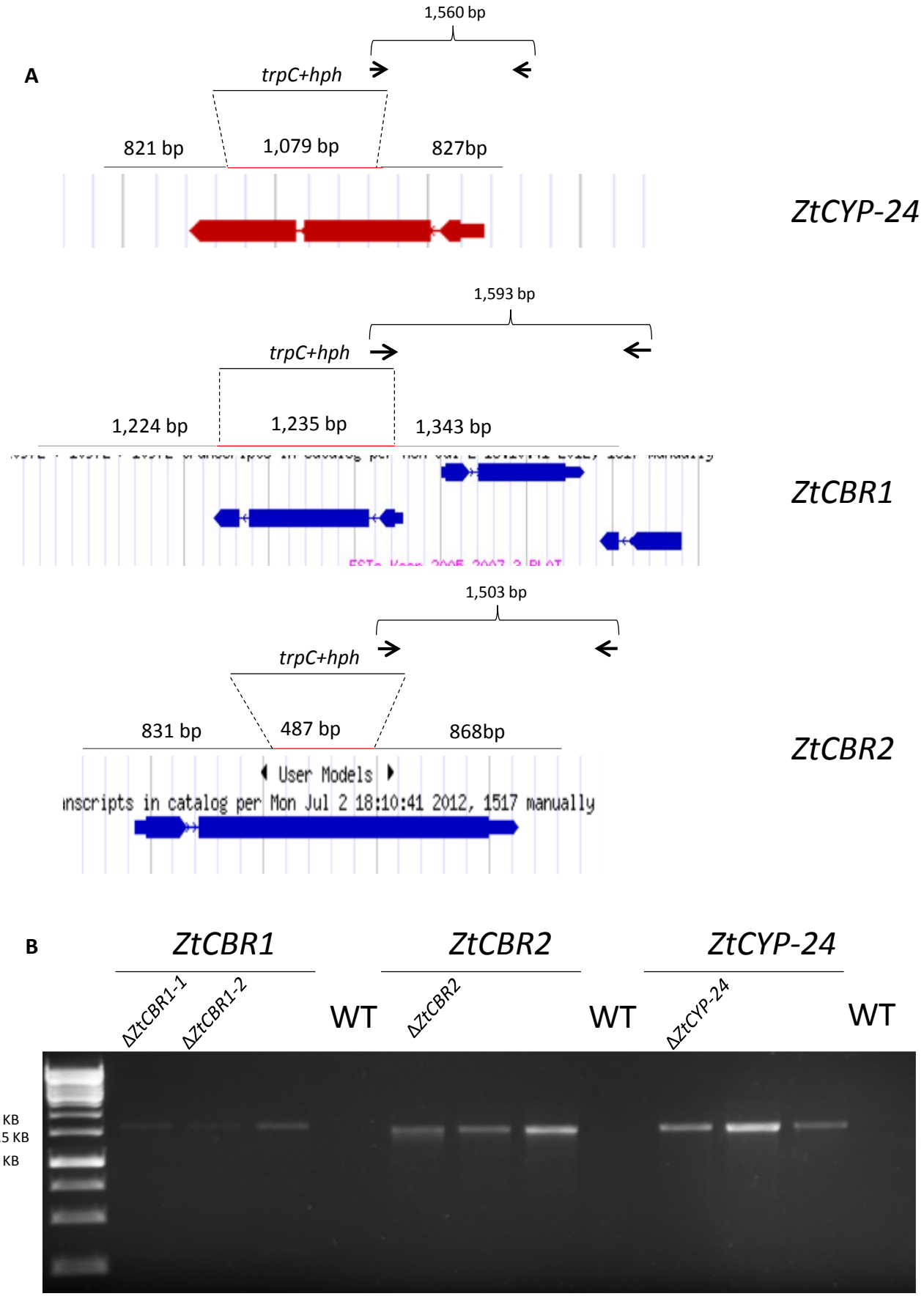
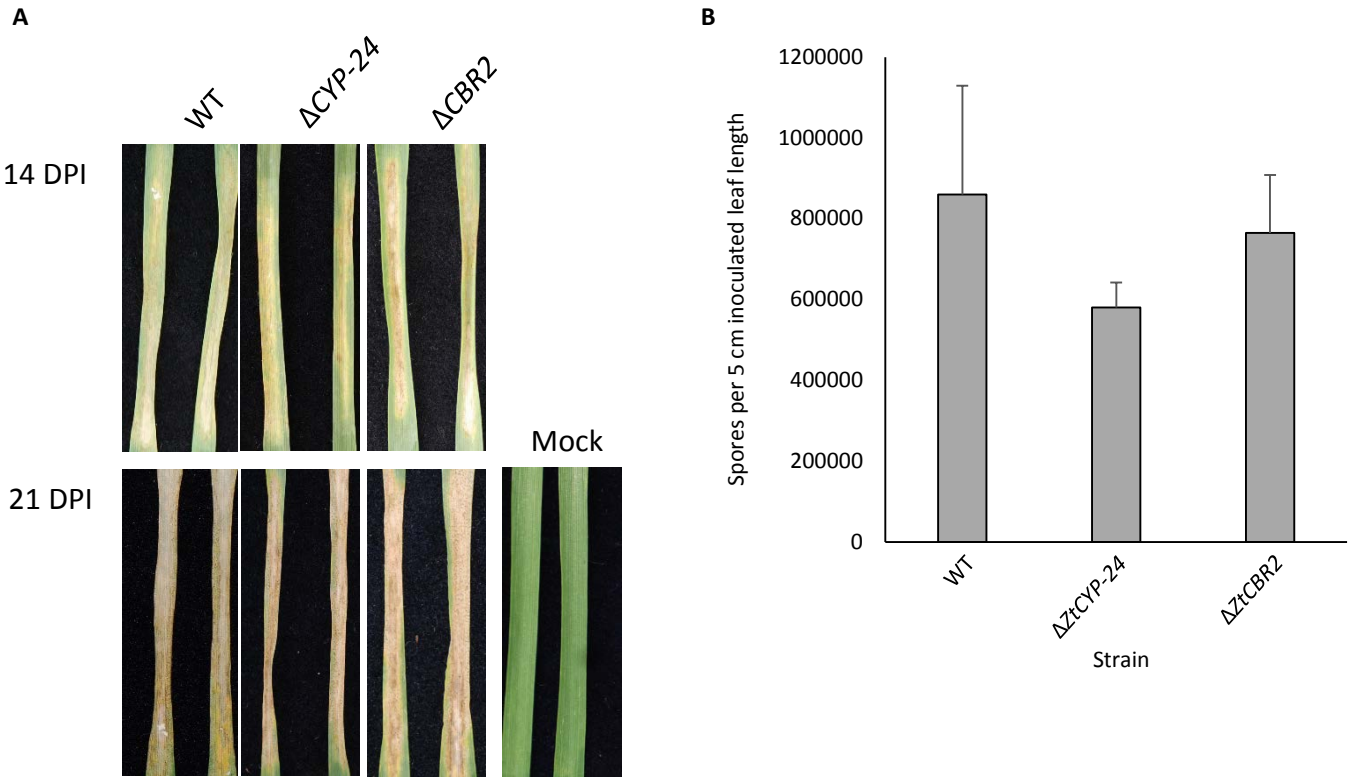



**Supplementary Figure 1. Targeted gene disruption strategy and confirmation.** (A) Positions of flanking sequences relative to the genes of interest in the *Z. tritici* genome, sizes of flanking sequences and sizes of regions to be replaced by the hygromycin resistance cassette under a *trpC* promoter (*trpC+hph*). Primers used for confirmation of successful gene disruption are marked with arrows and amplicon size is indicated above. (B) Confirmation of integration of *trpC+hph* at the correct location in the correct orientation using a primer from within *hph* and primer from within 200 bp of genomic DNA adjacent to one of the flanking sequences used to guide insertion. A single band of the correct amplicon size is indicative of successful gene targeting. WT gDNA was used as a control. Strains taken forward for further studies and their names are indicated above the relevant PCR lanes.




**Supplementary Figure 2. Wheat leaf infection assays with Δ*ZtCYP-24* and Δ*ZtCBR2* strains**

(A) Leaves infected with WT, Δ*ZtCYP-24* and Δ*ZtCBR2* after 14 and 21 DPI; mock-inoculated control leaves at 21 DPI are shown to the right. A total of 12 leaves per strain/mock were inoculated and two representative leaves are shown. (B) Mean number of spores (recovered by washing) per 5 cm length of inoculated leaf for WT, ΔZt*CYP-24* and Δ*ZtCBR2*.

| Primer name | Sequence | Use | Restriction site |
|---|---|---|---|
| NB5.5-1F | aaaagatctTTGTCATTCGTGTAGCCTGTGT | Amplification of flanking sequence NB5.5-1 for targeting *Ztcbr1* | BglII |
| NB5.5-1R | aaaggcgcgccactagtACTTGCTGGAGAGACTCTGCG | Amplification of flanking sequence NB5.5-1 for targeting *Ztcbr1* | SpeI |
| NB5.3-1F | aaaggcgccATCTGTGGTCCTCCTCCTATGGTT | Amplification of flanking sequence NB5.3-1 for targeting *Ztcbr1* | NarI |
| NB5.3-1R | aaaggcgcgcccAGGCAAAAAAGGCGTCTATGA | Amplification of flanking sequence NB5.3-1 for targeting *Ztcbr1* | SgsI |
| HYG-N1F | aaaactagtGATATTGAAGGAGCATTTTTTGG | Amplification of hygromycin resistance cassette to clone into pNOV2114 | SpeI |
| HYG-N1R | aaaggcgcccTACTACTATTCCTTTGCCCTCG | Amplification of hygromycin resistance cassette to clone into pNOV2114 | NarI |
| CBR2 F1 F | aagagctcACGATGAACGCATCGGTTTCATCACGATGCTACGGTTTTG | Amplification of flanking sequence CBR2F1 for targeting *Ztcbr2* | SacI |
| CBR2 F1 R | aaggtaccAAGTGAATTGCAGCCCACGCA | Amplification of flanking sequence CBR2F1 for targeting *Ztcbr2* | KpnI |
| CBR2 F2 F | aaaagcttGCCGACATTCGCGGTCCCAA | Amplification of flanking sequence CBR2F2 for targeting *Ztcbr2* | HindIII |
| CBR2 F2 R | aaactagtCGAGTACCGCGCTTTGGGGG | Amplification of flanking sequence CBR2F2 for targeting *Ztcbr2* | SpeI |
| CYP-24 F1 F | aaaagcttATGTCGAGAGATCGTGAGTTGTTTGCA | Amplification of flanking sequence CYP-24F`1 for targeting *Ztcyp-24* | HindIII |
| CYP-24 F1 R | aatctagaGATTCAACTTTGCACTGCTAGAAGTGAAGAT | Amplification of flanking sequence CYP-24F`1 for targeting Ztcyp-24 | XbaI |
| CYP-24 F2 F | aaggtaccGATGACTGCTGTTGATCTCCTTCGAA | Amplification of flanking sequence CYP-24F2 for targeting *Ztcyp-24* | KpnI |
| CYP-24 F2 R | aagagctcTTGCTTGAACACTTGGCTTTCGGT | Amplification of flanking sequence CYP-24F2 for targeting Ztcyp-24 | SacI |
| CBR1 + Hyg F | GCTGATCTGACCAGTTGCCT | CBR1 KO confirmation | |
| CBR1 + Hyg R | CAATCGTGAGGGGTTCAGCT | CBR1 KO confirmation | |
| CBR2 + Hyg F | AGTCGTCCAGGCGGTGAGCA | CBR2 KO confirmation | |
| CBR2 + Hyg R | GGCGAAGCTCTCCGGCCATC | CBR2 KO confirmation | |
| CYP-24 + Hyg F | AGTCGTCCAGGCGGTGAGCA | CYP-24 KO confirmation | |
| CYP-24 + Hyg R | GGCCCTCCCTGAACTCCCCA | CYP-24 KO confirmation | |

**Supplementary Table 1** All primers used in this study with added adenines followed by restriction sites in lower case at the 5’ end.

| Fungal genome | URL |
|---|---|
| *Zymoseptoria tritici* | http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html |
| *Ustilago maydis* | http://genome.jgi.doe.gov/Ustma1/Ustma1.home.html |
| *Stagonospora nodorum* | http://genome.jgi.doe.gov/Stano2/Stano2.home.html |
| *Puccinia graminis* | http://genome.jgi-psf.org/Pucgr1/Pucgr1.home.html |
| *Piriformospora indica* | http://genome.jgi-psf.org/Pirin1/Pirin1.home.html |
| *Neurospora tetrasperma* | http://genome.jgi-psf.org/Neute_matA2/Neute_matA2.home.html |
| *Neurospora crassa* | http://genome.jgi-psf.org/Neucr2/Neucr2.home.html |
| *Mycosphaerella fijiensis* | http://genome.jgi.doe.gov/Mycfi2/Mycfi2.home.html |
| *Fusarium graminearum* | http://genome.jgi.doe.gov/Fusgr1/Fusgr1.home.html |
| *Dothistroma septosporum* | http://genome.jgi-psf.org/Dotse1/Dotse1.home.html |
| *Colletotrichum graminicola* | http://genome.jgi-psf.org/Colgr1/Colgr1.home.html |
| *Cochliobolus sativus* | http://genome.jgi.doe.gov/Cocsa1/Cocsa1.home.html |
| *Cochliobolus heterostrophus* | http://genomeportal.jgi-psf.org/CocheC5_3/CocheC5_3.home.html |
| *Blumeria graminis* f. sp. *hordei* | http://genome.jgi.doe.gov/Blugr1/Blugr1.home.html |
| *Aspergillus terreus* | http://genome.jgi.doe.gov/Aspte1/Aspte1.home.html |
| *Aspergillus nidulans* | http://genome.jgi.doe.gov/Aspnid1/Aspnid1.home.html |
| *Aspergillus fumigatus* | http://genome.jgi.doe.gov/Aspfu1/Aspfu1.home.html |

**Supplementary Table 2** URLs of all genome sequences incorporated into genome-wide analysis of *CBR* genes.