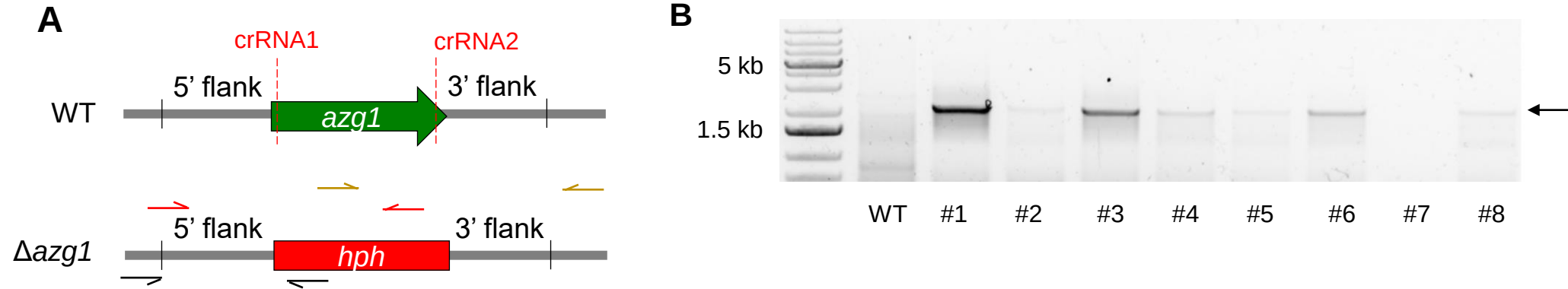
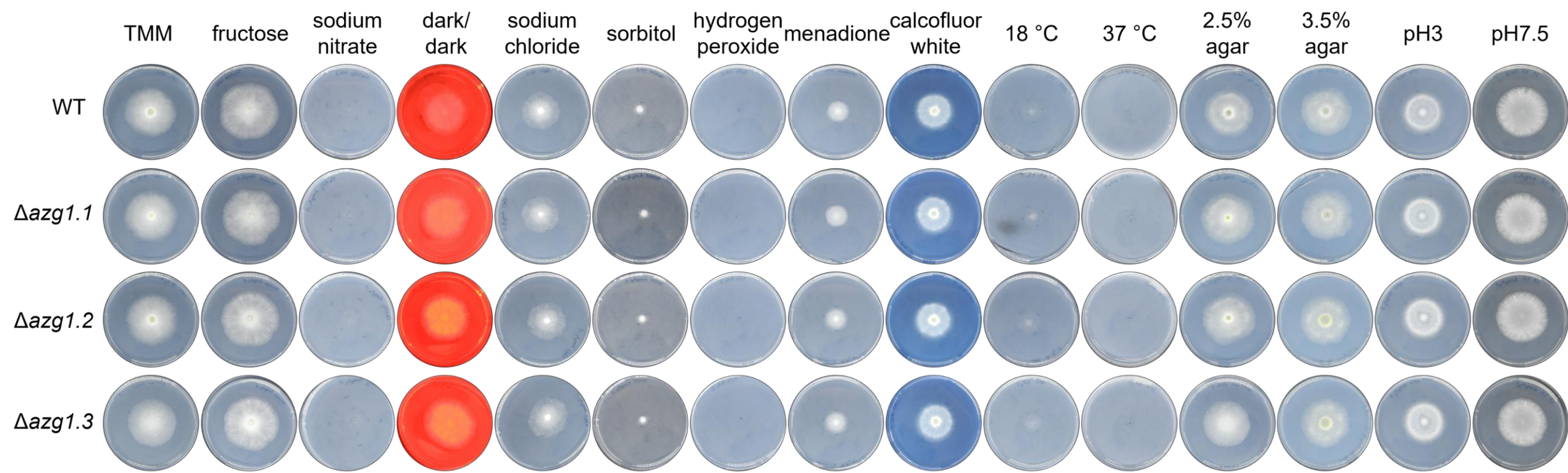


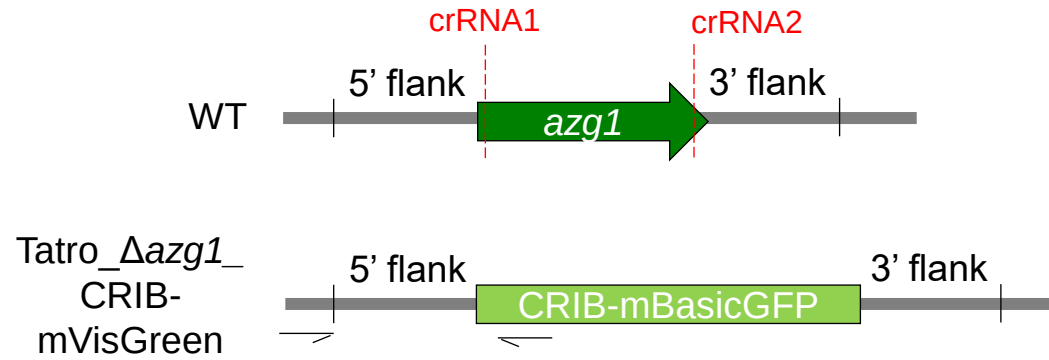
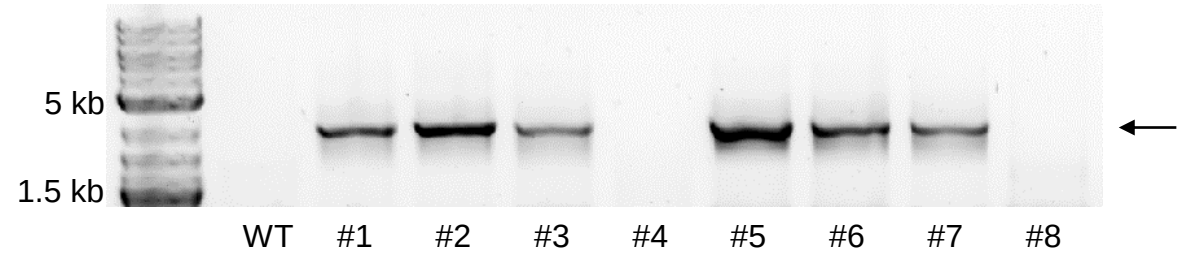
# **Supplementary Files**



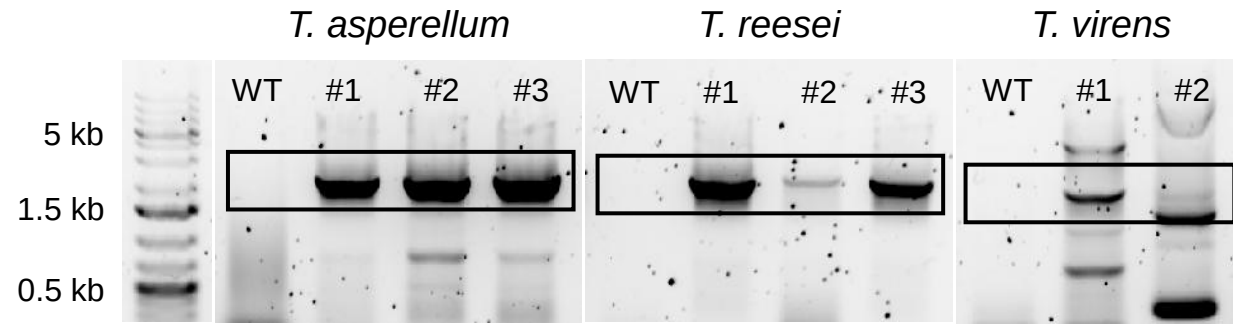
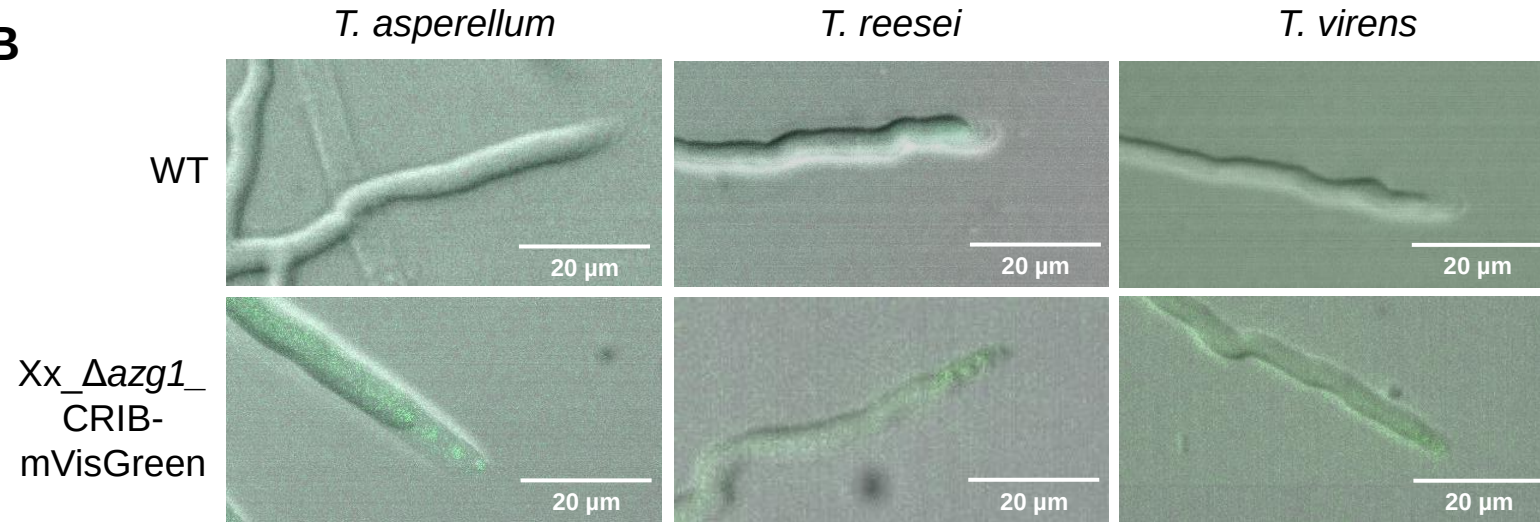
**Supplementary Figure S1.** Deletion of *azg1* in *T. atroviride*. (A) Scheme of *azg1* deletion strategy, highlighting the position of the cutting sites (crRNAs) for the CRISPR/Cas9 system. The 1 kb upstream and downstream regions (5' and 3' flanks) were used for homology directed recombination. Integration efficiency of the *hph* cassette was enhanced by using a split-marker strategy. The primers used to generate the two separate, but partially overlapping fragments that together form the marker cassette (Supplementary Table S2) are highlighted in red (fragment 1) or gold (fragment 2). In black are reported the primers used for genotyping of the resulting mutants (Supplementary Table S2). (B) Visualization of the products resulting from PCR of the 8 transformants in a 1% agarose gel. The black arrow on the right indicates the expected fragment size.



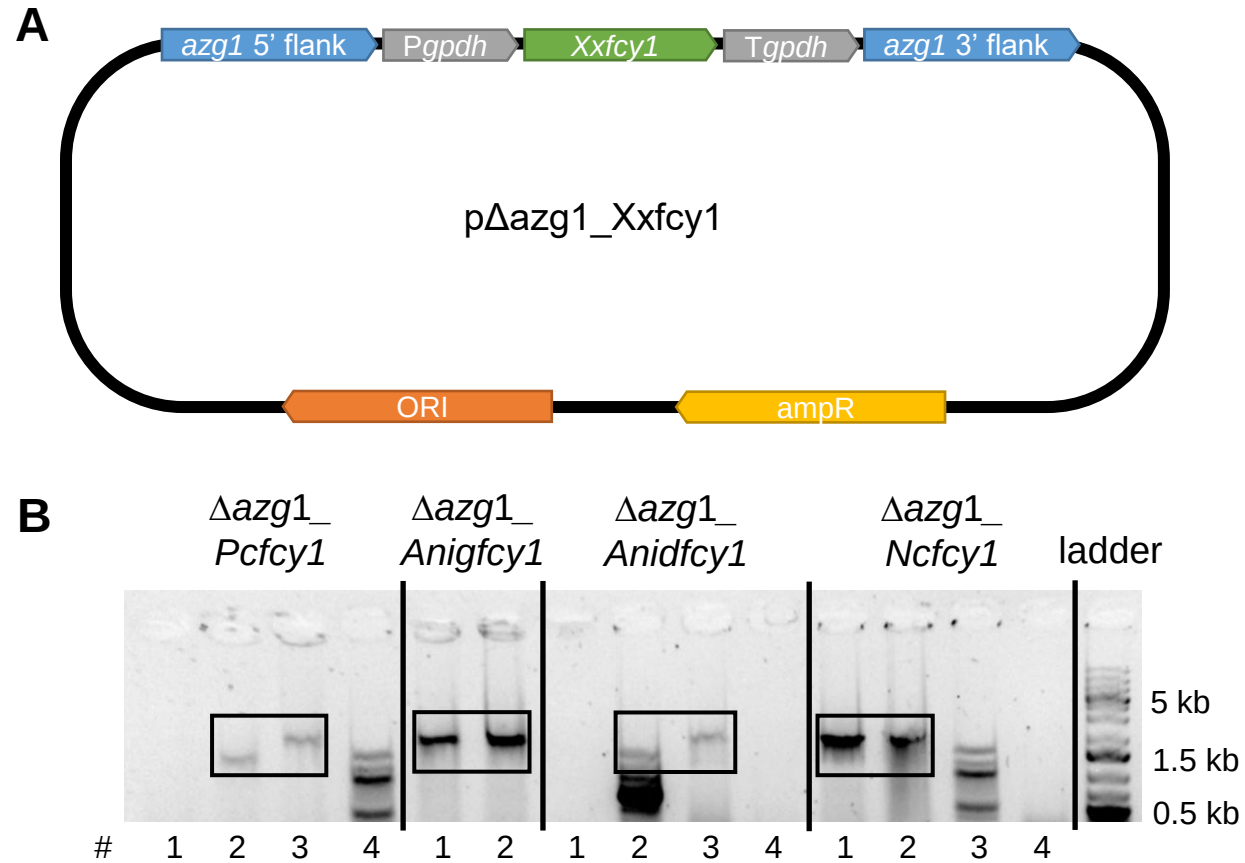
**Supplementary Figure S2.** Phenotypic characterization of *azg1* deletion mutant in *T. atroviride*. (A) Picture of  $\Delta azg1$  strains in comparison to the WT after 3 days incubation. Standard growth condition, unless differently specified, consisted of 25 °C for 12 h light and 12 h dark, in TMM containing 1% glucose and 10 mM glutamine as carbon and nitrogen sources, respectively. 1% fructose or 10 mM sodium nitrate were used to test growth with different nutrient sources. The dark/dark condition indicated complete absence of light. 0.5 M sodium chloride, 1.5 M sorbitol, 20 mM hydrogen peroxide, 0.2 mM menadione or 50  $\mu$ g/mL calcofluor white were added to the medium as stressors. At last, different temperature, agar concentration and pH conditions were tested.

**A****B**

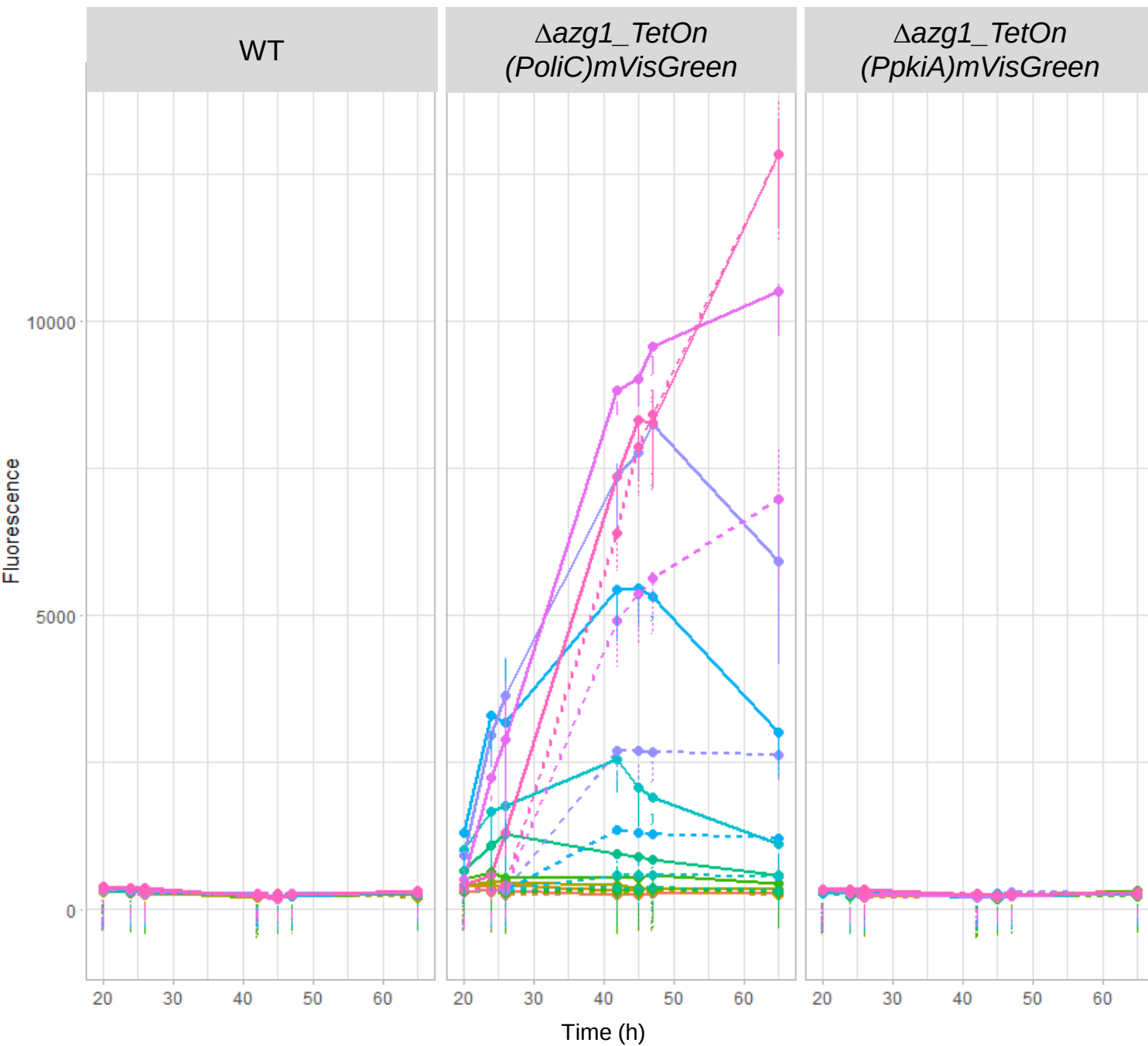
**Supplementary Figure S3.** *azg1* as counter-selectable marker in *T. atroviride*. (A) Schematic representation of reporter integration strategy into *azg1* locus, highlighting the position of the cutting sites (crRNAs) for the CRISPR/Cas9 system, the 1 kb upstream and downstream regions (5' and 3' flanks) used for homology directed recombination and, in black, the primers used for genotyping of the resulting mutants (Supplementary Table S2). (B) Visualization of the products resulting from PCR genotyping PCR for 8 transformants in a 1% agarose gel. The black arrow on the right indicates the expected fragment size.

**A****B**

**Supplementary Figure S4.** *azg1* as counter-selectable marker in other *Trichoderma* species. (A) Visualization of the products resulting from genotyping PCR for the transformants obtained in *T. asperellum*, *T. reesei* and *T. virens* in a 1% agarose gel. The black rectangles highlights the area for expected fragment size of about 2 kb. (B) Microscopy pictures of one representative clones per species in comparison to the respective WT confirming the integration and functionality of the CRIB-mBasicGFP reporter in *T. asperellum*, *T. reesei* and *T. virens* mutant strains and the potential of *azg1* locus as negative selectable marker. The Xx in front of the mutant strains name indicates that different background species are available (e.g. *T. asperellum*, *T. reesei* and *T. virens*)



**Supplementary Figure S5.** Induction of 5-FC sensitivity in *T. atroviride*. (A) Generic plasmid map showing the essential components for the integration of the heterologous cytosine deaminase-encoding sequence (*Xxfcy1*) in the *azg1* locus of *T. atroviride* P1. The 1 kb *azg1* upstream and downstream regions (*azg1* 5' and 3' flanks) were used for homology directed recombination. The promoter and terminator regions of *T. reesei gpdh* were used to drive gene expression. Origin of replication (ORI) and ampicillin resistance (*ampR*) were used for plasmid mplification in *E. coli*. (B) Visualization in a 1% agarose gel of the products produced by PCR genotyping from the transformants generated by replacing *azg1* with the heterologous cytosine deaminase-encoding sequence from four different fungal species: *P. chrysogenum* (*Pcfcy1*), *A. niger* (*Anigfcy1*), *A. nidulans* (*Anidfcy1*) and *N. crassa* (*Ncfcy1*). The expected band is highlighted for each specific construct.



**Supplementary Figure S6.** Evaluation of doxycycline effects on fungal growth. Background adjusted mean fluorescence signal over time (20 - 64 h) of *T. atroviride* WT,  $\Delta azg1\_TetOn(PoliC)mVisGreen$  and  $\Delta azg1\_TetOn(PpkiA)mVisGreen$  in the presence of different doxycycline concentration. T(0) (continuous lines) indicates measurements for samples where the doxycycline was added immediately after inoculation, while T(1) (dashed lines) samples where doxycycline was added after 20 h of incubation at 25 °C

Doxycycline added

— 0d

- - - 1d

Doxycycline ( $\mu g/mL$ )

— 0

— 0.390625

— 0.78125

— 1.5625

— 3.125

— 6.25

— 12.5

— 25

— 50

— 100

**Supplementary Table S1.** List of strains used in this study

| Name                                       | Genotype                                                                                      | Origin                                |
|--------------------------------------------|-----------------------------------------------------------------------------------------------|---------------------------------------|
| <i>E. coli</i> Stellar                     | <i>E. coli</i> HST08 (for complete genotype refer to manufacturer homepage)                   | Takara, Saint-Germain-en-Laye, France |
| NEB 5-alpha <i>E. coli</i> competent cells | <i>E. coli</i> DH5 $\alpha$ derivative (for complete genotype refer to manufacturer homepage) | New England Biolabs, Ipswich, MA, USA |
| <i>T. atroviride</i> P1                    | <i>T. atroviride</i> WT isolate                                                               | ATCC 74058                            |
| $\Delta$ azg1                              | <i>T. atroviride</i> P1 $\Delta$ azg1::hph                                                    | This study                            |
| <i>B. cinerea</i> B05.10                   | <i>B. cinerea</i> WT isolate                                                                  | ATCC 11542                            |
| <i>F. graminearum</i> FG 8/WT-1            | <i>F. graminearum</i> WT isolate                                                              | Jansen et al. 2005                    |
| Tatro $\Delta$ azg1_CRIB_mVisGreen         | <i>T. atroviride</i> P1 $\Delta$ azg1::Ppki_CRIB_mBasicgfp_Tcbh2                              | This study                            |
| <i>T. reesei</i> QM6a                      | <i>T. reesei</i> WT isolate                                                                   | ATCC 13631                            |
| <i>T. virens</i> Gv29-8                    | <i>T. virens</i> WT isolate                                                                   | FGSC 10586                            |
| <i>T. asperellum</i> CBS 433.97            | <i>T. asperellum</i> WT isolate                                                               | ATCC 204424                           |
| Trees $\Delta$ azg1_CRIB_mVisGreen         | <i>T. reesei</i> QM6a $\Delta$ azg1::Ppki_CRIB_mBasicgfp_Tcbh2                                | This study                            |
| Tvir $\Delta$ azg1_CRIB_mVisGreen          | <i>T. virens</i> Gv29-8 $\Delta$ azg1::Ppki_CRIB_mBasicgfp_Tcbh2                              | This study                            |
| Tasp $\Delta$ azg1_CRIB_mVisGreen          | <i>T. asperellum</i> CBS 433.97 $\Delta$ azg1::Ppki_CRIB_mBasicgfp_Tcbh2                      | This study                            |
| <i>P. chrysogenum</i>                      | <i>P. chrysogenum</i> WT strain                                                               | ATCC 10106                            |
| <i>A. niger</i>                            | <i>A. niger</i> WT isolate                                                                    | ATCC 1015                             |
| <i>A. nidulans</i>                         | <i>A. nidulans</i> $\Delta$ thi4                                                              | FGSC A4                               |
| <i>N. crassa</i>                           | <i>N. crassa</i> WT isolate                                                                   | OR47A                                 |
| $\Delta$ azg1_Pcfcy1                       | <i>T. atroviride</i> P1 $\Delta$ azg1::Pcfcy1                                                 | This study                            |
| $\Delta$ azg1_Anigfcy1                     | <i>T. atroviride</i> P1 $\Delta$ azg1::Anigfcy1                                               | This study                            |
| $\Delta$ azg1_Anidfcy1                     | <i>T. atroviride</i> P1 $\Delta$ azg1::Anidfcy1                                               | This study                            |
| $\Delta$ azg1_Ncfcy1                       | <i>T. atroviride</i> P1 $\Delta$ azg1::Ncfcy1                                                 | This study                            |
| $\Delta$ azg1_TetOn(PoliC)mVisGreen        | <i>T. atroviride</i> P1 $\Delta$ azg1::TetOn(PoliC)mBasicgfp_Tcbh2                            | This study                            |
| $\Delta$ azg1_TetOn(PpkiA)mVisGreen        | <i>T. atroviride</i> P1 $\Delta$ azg1::TetOn(PpkiA)mBasicgfp_Tcbh2                            | This study                            |
| $\Delta$ azg1_mKO2                         | <i>T. atroviride</i> P1 $\Delta$ azg1::mKO2opt                                                | This study                            |
| $\Delta$ azg1_mTagBFP2opt                  | <i>T. atroviride</i> P1 $\Delta$ azg1::mTagBFP2opt                                            | This study                            |
| $\Delta$ azg1_K2S                          | <i>T. atroviride</i> P1 $\Delta$ azg1::Katushka2S                                             | This study                            |

**Supplementary Table S3.** List of plasmids used in this study

| Plasmid                              | Origin                   |
|--------------------------------------|--------------------------|
| pΔazg1-HPH                           | This study               |
| pGFP-XYR1                            | Lichius et al., 2014     |
| Tatro_pΔazg1-CRIB-mVisGreen          | This study               |
| Trees_pΔazg1-CRIB-mVisGreen          | This study               |
| Tvir_pΔazg1-CRIB-mVisGreen           | This study               |
| Tasp_pΔazg1-CRIB-mVisGreen           | This study               |
| pLH2-Tasp                            | Moreno-Ruiz et al., 2021 |
| pΔazg1_Pcfcy1                        | This study               |
| pΔazg1_Anigfcy1                      | This study               |
| pΔazg1_Anidfcy1                      | This study               |
| pΔazg1_Ncfcy1                        | This study               |
| pΔazg1 Tet-On(PoliC) mVisGreen       | This study               |
| pΔazg1 Tet-On(Ppkia) mVisGreen       | This study               |
| pΔfcyB sGFP <sup>Tet-On(PoliC)</sup> | Baldin et al., 2022      |
| pΔfcyB sGFP <sup>Tet-On(Ppkia)</sup> | Baldin et al., 2022      |
| pΔazg1_PgpdH mko2opt                 | This study               |
| pΔazg1_PgpdH mtagbfp2opt             | This study               |
| pΔazg1_PgpdH k2s                     | This study               |
| pBM12                                | Storer et al., 2024      |
| pBM123                               | Storer et al., 2024      |
| pFG39                                | Storer et al., 2024      |

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