**Supplementary Information**

# Regulation of TRI5 expression and deoxynivalenol biosynthesis by a long non-coding RNA in *Fusarium graminearum*

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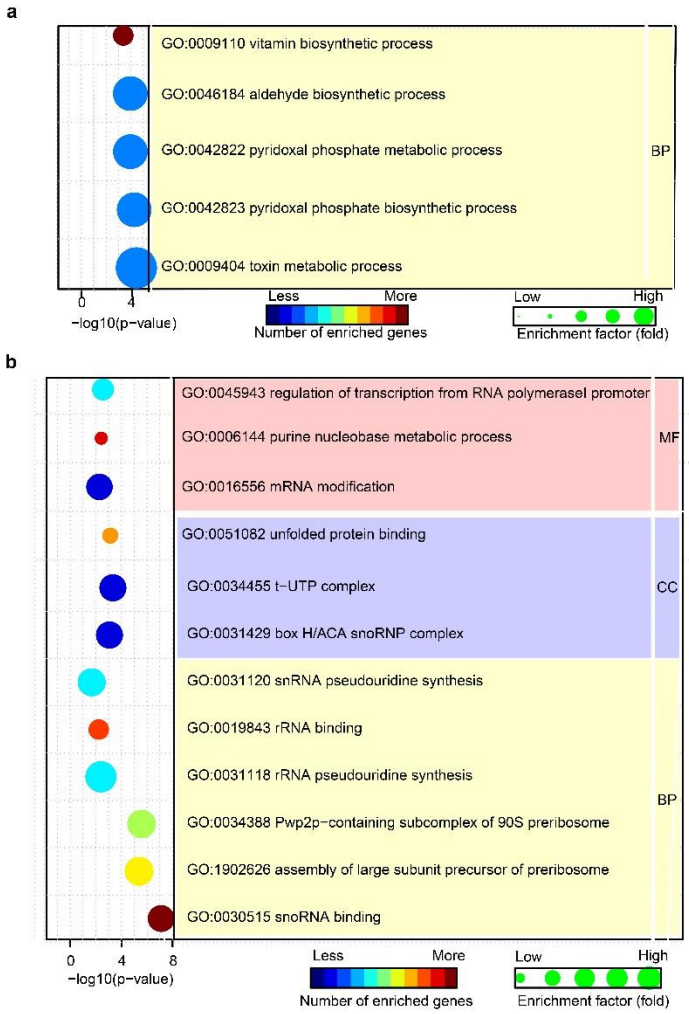
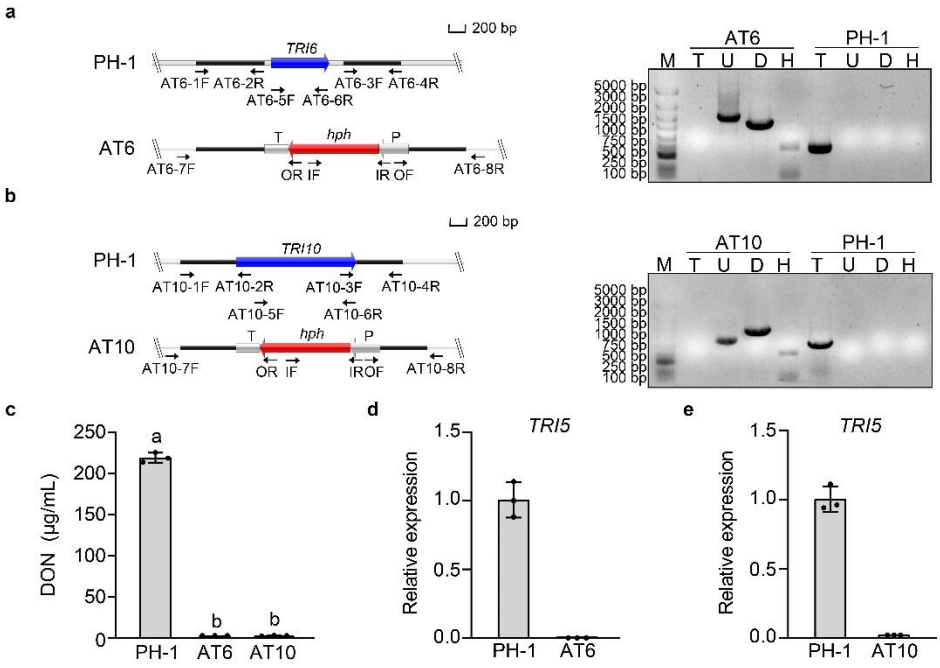
## I. Supplementary Figures




**Supplementary Fig. 1 GO enrichment analysis with DEGs in the *tri6* and *tri10* mutants.**

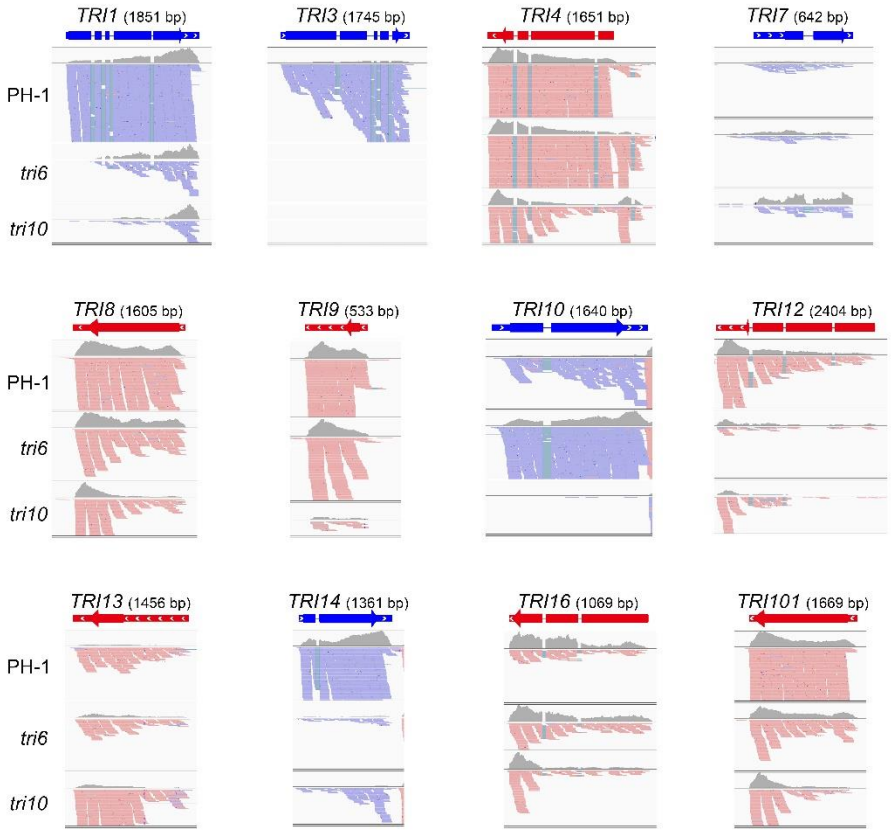
**a** Enriched gene ontology (GO) terms in genes with down-regulated transcripts in the *tri6* mutant. **b** Enriched gene ontology (GO) terms in genes with down-regulated transcripts in the *tri10* mutant. MF, molecular function; CC, cell component; BP, biological process.




**Supplementary Fig. 2 Generation of the *tri6* and *tri10* gene replacement mutants by an inverted *hph* cassette.**

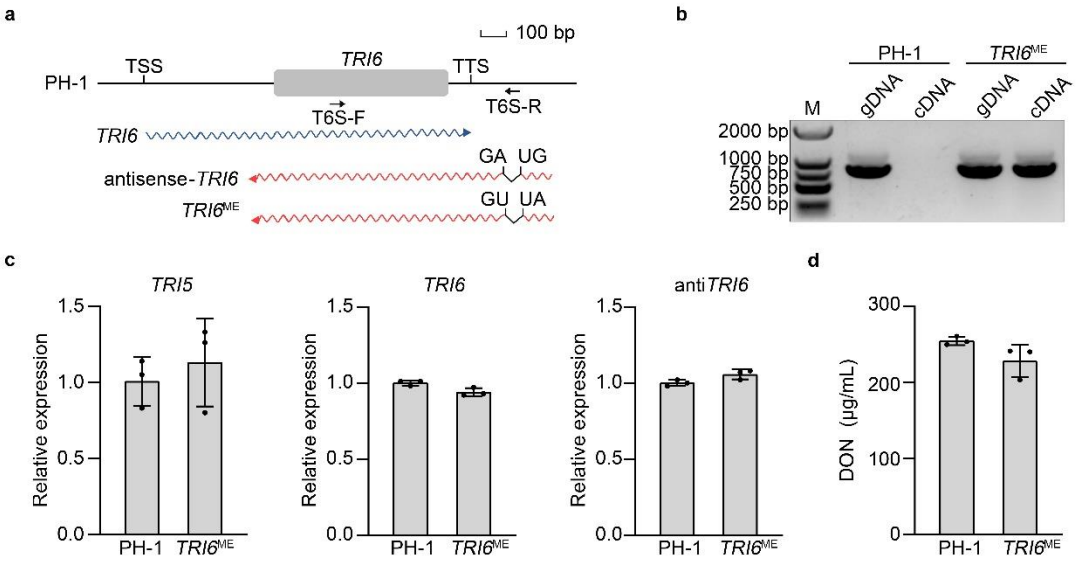
**a** Schematic drawing of the primers used to generate gene replacement transformants AT6 and screen for knockout mutants (left panel). The upstream and downstream flanking sequences of the *TRI6* gene were amplified with primer pairs AT6-1F/AT6-2R and AT6-3F/AT6-4R and connected to the inverted *hph* cassette to generate gene replacement constructs. P, TrpC promoter; T, CaMV ployA signal terminator. DNA isolated from the wild-type strain PH-1 and *tri6* mutant (AT6) for the PCR verification of *TRI6* deletion (right panel). PCR products amplified by primer pairs AT6-7F/OR (lane U) and OF/AT6-8R (lane D) showed the occurrence of homologous recombination at the upstream and downstream flanking sequences of the *TRI6* gene, respectively. Lane T was amplified with primer pairs AT6-5F and AT6-6R to show the deletion of *TRI6* genes. Lane H showed the amplification of the inverted *hph* gene with primers IF and IR. M, molecular marker. **b** Schematic drawing of the primers used to generate gene replacement transformants AT10 and screen for knockout mutants (left panel). The upstream and downstream flanking sequences of the *TRI10* gene were amplified with primer pairs AT10-1F/AT10-2R and AT10-3F/AT10-4R and connected to the inverted *hph* cassette to generate gene replacement constructs. DNA isolated from the wild-type strain PH-1 and *tri10* mutant (AT10) for the PCR verification of *TRI10* deletion (right panel). PCR products amplified by primer pairs AT10-7F/OR (lane U) and OF/AT10-8R (lane D) showed the occurrence of homologous recombination at the upstream and downstream flanking sequences of the *TRI10* gene, respectively. Lane T was amplified with primer pairs AT10-5F and AT10-6R to show the deletion of *TRI10* genes. Lane H showed the amplification of the inverted *hph* gene with primers IF and IR. M, molecular marker. **c** Deoxynivalenol (DON) levels in 7-day-old LTB cultures of wild-type

PH-1, AT6, and AT10 transformants. **d** Assayed for the expression of *TRI5* by qRT-PCR with RNA isolated from 3-day-old LTB cultures of the wild-type PH-1 and AT6. **e** Assayed for the expression of *TRI5* by qRT-PCR with RNA isolated from 3-day-old LTB cultures of the wild-type PH-1 and AT10. For each gene, its expression level in PH-1 was arbitrarily set to 1. For **c**, **d**, and **e**, mean and standard deviation were estimated with data from three ($n = 3$) independent replicates (marked with black dots on the bars). For DON production, different letters indicate significant differences based on the one-way ANOVA followed by Turkey's multiple range test. Differences were considered statistically significant when $p$-value is $< 0.05$. The exact $p$-values are shown in the Source Data file.




**Supplementary Fig. 3 Expression profiles of the *TRI* genes without antisense transcripts.**

Predicted ORFs of marked *TRI* genes and read counts of their transcripts in RNA-seq data of PH-1 and the *tri6* and *tri10* deletion mutants. No antisense transcripts of these *TRI* genes were observed. The length of transcripts was indicated in the figure.

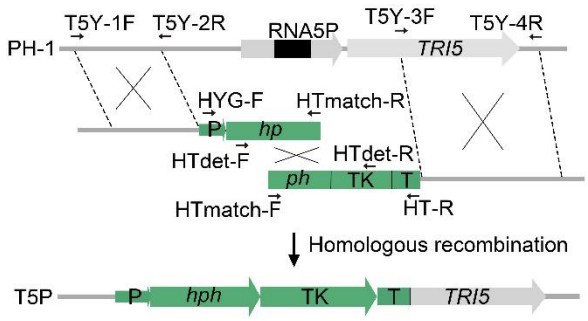



**Supplementary Fig. 4 The antisense transcripts of *TRI6*.**

**a** Schematic drawing of the *TRI6* and *TRI6*$^{ME}$ alleles and its sense and antisense transcripts. Specific primers for PCR verification were indicated. **b** Verification by PCR through specific primer T6S-F/T6S-R. RNA isolated from 3-day-old LTB cultures of PH-1 was used for cDNA synthesis. Mutations on the splicing site disrupted the intron splicing in the *TRI6*$^{ME}$ strain. M, molecular marker. **c** Relative expression levels of *TRI5*, *TRI6*, and antisense-*TRI6* were assayed by qRT-PCR with RNA isolated from 3-day-old LTB cultures of PH-1 and *TRI6*$^{ME}$ transformant. For each gene, its expression level in PH-1 was arbitrarily set to 1. **d** DON production in 7-day-old LTB cultures of PH-1 and *TRI6*$^{ME}$ transformant. For **c** and **d**, mean and standard deviation were estimated with data from three ($n$ = 3) independent replicates (marked with black dots on the bars). For DON production, no significant statistical differences were observed based on the two-tailed unpaired $t$ test. The exact $p$-values are shown in the Source Data file.

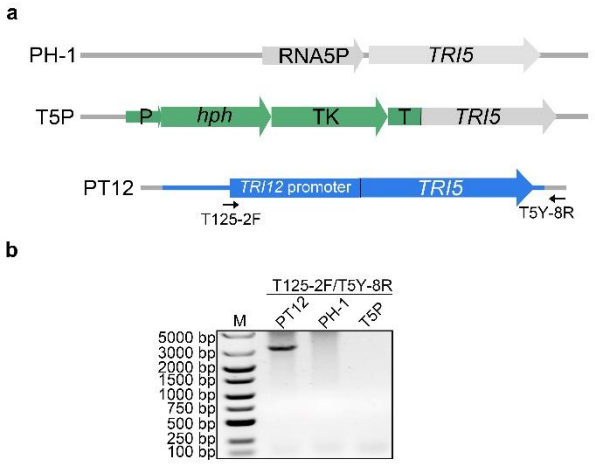

**Supplementary Fig. 5 Full-length transcript of RNA5P.**

Two isoforms of RNA5P are formed due to alternative splicing of the intron and are predicted to encode two small proteins that share the same stop codon. Two isoforms of RNA5P were underlined in red and blue, respectively. The GU-AG splicing sites were indicated with red font. The red arrow marks 3×FLAG.




**Supplementary Fig. 6 Schematic drawing of the primers used to generate gene replacement transformants T5P (the *TRI5*$^{\Delta promoter}$ mutant).**

The upstream and downstream flanking sequences of the *TRI5* gene were amplified with labelled primer pairs and connected to the hygromycin phosphotransferase (*hph*)-thymidine kinase (TK) fusion cassette to generate the *TRI5*$^{\Delta promoter}$ mutant (T5P). P, TrpC promoter; T, CaMV ployA signal terminator.




**Supplementary Fig. 7 PCR verification of the $P_{TRI12}$-*TRI5* transformants.**

**a** Schematic drawing of RNA5P in the upstream region of *TRI5* and the generation of the $TRI5^{\Delta promoter}$ (T5P) and $P_{TRI12}$-*TRI5* (PT12) transformants. **b** PCR products were amplified with primer pairs T125-2F and T5Y-8R from genomic DNA isolated from the wild-type strain PH-1, T5P ($TRI5^{\Delta promoter}$ transformant), and PT12 ($P_{TRI12}$-*TRI5* transformant). PCR products amplified from the DNA templates of PT12 transformant showed the replacement of the *TRI5* promoter with that of *TRI12*. DNA template isolated from the wild-type PH-1 (WT) and T5P ($TRI5^{\Delta promoter}$ transformant) was used as a control. M, molecular marker.

## II. Supplementary Tables

**Supplementary Table 1. Strains of *Fusarium graminearum* used or generated in this study.**

| Strains | Brief description | References |
|---|---|---|
| PH-1 | Wild-type strain of *F. graminearum* | (Cuomo et al., 2007) |
| *tri6* | The *tri6* deletion mutant of PH-1 | (Seong et al., 2009) |
| *tri10* | The *tri10* deletion mutant of PH-1 | (Seong et al., 2009) |
| *TRI6*$^{AD}$ | Deletion of the promoter of anti*TRI6* in PH-1 | This study |
| *tri5* | The *tri5* deletion mutant of PH-1 | This study |
| RAT1 | $P_{RP27}$-anti*TRI5* transformant of PH-1 | This study |
| RAT2 | $P_{RP27}$-anti*TRI5* transformant of PH-1 | This study |
| L3F | RNA5P-3×FLAG-$T_{CaWV}$ transformant of PH-1 | This study |
| EF1 | *ESA1*-3×FLAG transformant of PH-1 | (Jiang et al., 2020) |
| T5P | Deletion of the promoter of *TRI5* in PH-1 | This study |
| RNA5P$^{M}$ | Transformant with a nucleotide inserted in the RNA5P | This study |
| PT12 | $P_{TRI12}$-*TRI5* transformant of T5P | This study |
| LT7 | $P_{RP27}$-RNA5P-$T_{CaWV}$ transformant of PH-1 | This study |
| LT10 | $P_{RP27}$-RNA5P-$T_{CaWV}$ transformant of PH-1 | This study |
| TR1 | $P_{TrpC}$-RNA5P transformant of T5P | This study |
| TR2 | $P_{TrpC}$-RNA5P transformant of T5P | This study |
| ATR1 | $P_{TrpC\text{-}inverted}$-RNA5P transformant of T5P | This study |
| ATR3 | $P_{TrpC\text{-}inverted}$-RNA5P transformant of T5P | This study |
| TM6 | Transformant with GTGA to TGAG mutation in the promoter of RNA5P | This study |
| TM66 | Transformant with GTGA to TGAG mutation in the promoter of RNA5P | This study |
| AT6 | The *tri6* deletion mutant of PH-1 with the replacement by reverse-transcribed *hph* cassette | This study |
| AT10 | The *tri10* deletion mutant of PH-1 with the replacement by reverse-transcribed *hph* cassette | This study |
| *TRI6*$^{ME}$ | Transformant of *TRI6*$^{AD}$ with the mutation on the splicing sites of *antiTRI6* | This study |

**Supplementary Table 2. DEGs up- or down-regulated in both the *tri6* and *tri10* mutants.**

| Up-regulated DEGs | |
|---|---|
| **Gene ID** | **Annotation** |
| FG1G13350 | Hypothetical protein |
| FG1G17480 | Hypothetical protein |
| FG1G36450 | Nik-1 protein |
| FG1G36460 | Isoprenylcysteine carboxyl methyltransferase |
| FG1G50780 | Hypothetical protein |
| FG2G10860 | Hypothetical protein |
| FG2G12010 | Glucose-repressible protein Grg1 |
| FG2G12090 | MFS transporter |
| FG2G13970 | Cryptochrome DASH |
| FG3G19710 | Extracellular serine-rich protein |
| FG3G21560 | Heam oxygenase-like protein |
| FG3G30990 | Kes1 protein |
| FG4G20830 | Allergen Fus c 3 protein |
| FG4G23950 | Hypothetical protein |

| Down-regulated DEGs | |
|---|---|
| **Gene ID** | **Annotation** |
| FG1G00280 | O-methylsterigmatocystin oxidoreductase |
| FG1G01160 | *TRI1* |
| FG1G02270 | Hypothetical protein |
| FG2G09410 | Phosphoenolpyruvate carboxykinase |
| FG2G20970 | Aminoglycoside phosphotransferase protein |
| FG2G26160 | *TRI3* |
| FG2G26190 | *TRI5* |
| FG2G26220 | *TRI11* |
| FG2G26240 | *TRI12* |
| FG2G26260 | *TRI14* |
| FG3G00040 | Integral membrane protein PTH11 |
| FG3G04840 | Pyridoxine biosynthesis protein PDX1 |
| FG3G04850 | Glutamine amidotransferase |
| FG4G08380 | Aurora protein kinase |
| FG4G21810 | Hypothetical protein |
| FG4G22230 | Omega-6 fatty acid desaturase |
| FG4G22330 | *TRI101* |

**Supplementary Table 3. The TPM (Transcripts Per Million) values of RNA5P, *TRI5*, anti*TRI5*, *TRI6*, anti*TRI6*, and a housekeeping gene *ACTIN* in PH-1.**

| RNA5P | *TRI5* | anti*TRI5* | *TRI6* | anti*TRI6* | *ACTIN* |
|---|---|---|---|---|---|
| 1.68 | 175.55 | 3.81 | 27.92 | 8.66 | 203.98 |