A Pathogenicity Accessory Chromosome in Fusarium oxysporum f. sp. vasinfectum

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Abstract

Members of the *Fusarium oxysporum* species complex are known to harbor accessory chromosomes that play critical roles in virulence and host adaptation. These chromosomes have the ability to be horizontally transferable between fungal isolates, posing a potential threat due to the spread of pathogenic traits.

In this study, we investigated a highly virulent *Fusarium oxysporum* f. sp. *vasinfectum* (Fov) strain, 89-1A, and identified a unique accessory chromosome containing a gene cluster that encodes a lactamase which has been confirmed to be a virulence factor for cotton.

A GFP tag was introduced into this chromosome, and two single-spore isolates carrying the GFP-tagged chromosome were obtained. A hydroponic test tube virulence assay confirmed the GFP insertion did not affect the virulence of strain 89-1A.

Using one of the GFP-tagged isolates, we performed flow cytometry screening and successfully isolated 44 conidia lacking GFP fluorescence, indicating they may have spontaneously lost the target chromosome. Subsequent virulence assays revealed isolates with a marked reduction in virulence.

Sequence analysis of the accessory chromosome identified eight genes potentially involved in host specificity. Among them, qPCR analysis showed that **FUN_000082** and **FUN_001597** were significantly upregulated—by approximately 200-fold and 60-fold, respectively—at 15 hours post-infection of cotton.

Ongoing research will employ molecular biology techniques and virulence assays to further elucidate the functions of these two genes. By identifying key genes and clarifying their roles during infection, this study provides novel insights into the function of pathogenicity-related accessory chromosomes, and may offer potential strategies for developing effective and sustainable measures to manage cotton diseases and safeguard crop yield.

Chapter 1: Genome Quality and Annotation Validation

This chapter ensures that the genome sequence used in the project is of high quality and that the gene annotation is accurate and reliable.

I. Extracting High-Quality Protein and CDS Sequences

We obtained the genome sequence of *Fusarium oxysporum* strain Fov891A using the PacBio sequencing platform. The lab already had the assembled genome file (`Fov891A.contigs.fasta`), the gene annotation file (`augustus_Fov891A.gff`), and the initial predicted protein file (`au_Fov891A.proteins.fa`).

To improve the quality of the predicted protein sequences, I used the genome and GFF files to extract high-confidence protein sequences and corresponding CDS (coding DNA sequences) using the tool `gffread`. This step was performed on our university's high-performance computing (HPC) cluster.

Code used

```
module load gffread

gffread augustus_Fov891A.gff \
  -g Fov891A.contigs.fasta \
  -y Fov891A_predicted_proteins.faa \
  -x Fov891A_predicted_CDS.fna
```

Conclusion

This process generated two output files: `Fov891A_predicted_proteins.faa` and `Fov891A_predicted_CDS.fna`, which were subsequently used for BUSCO analysis to evaluate the completeness of the genome annotation.

II. Assessing Protein Annotation Completeness Using BUSCO

To evaluate the completeness and reliability of the predicted protein sequences, BUSCO (Benchmarking Universal Single-Copy Orthologs) was used. I ran BUSCO in proteins mode with the sordariomycetes_odb10 lineage dataset on the HPC cluster.

Code used

```
nano run_busco_predicted.sh
```

```
#!/bin/bash
#PBS -N busco_predicted
#PBS -o busco_predicted_output.log
#PBS -e busco_predicted_error.log
#PBS -l nodes=1:ppn=4
#PBS -l walltime=01:00:00
#PBS -l mem=8gb
#PBS -V
#PBS -q medium

cd "$PBS_O_WORKDIR"

module load busco/5.4.3

busco -i Fov891A_predicted_proteins.faa \
      -o busco_predicted_output \
      -l sordariomycetes_odb10 \
      -m proteins \
      --cpu 4 \
      -f
```

```
qsub run_busco_predicted.sh
```

III. Reviewing BUSCO Output

After the BUSCO job finishes, I checked the summary results using the command below:

```
cat busco_predicted_output/short_summary*.txt
```

BUSCO Output

```
***** Results: *****

C:98.8%[S:97.9%,D:0.9%], F:0.4%, M:0.8%, n:3817
3772    Complete BUSCOs (C)
3738    Complete and single-copy BUSCOs (S)
34      Complete and duplicated BUSCOs (D)
16      Fragmented BUSCOs (F)
29      Missing BUSCOs (M)
3817    Total BUSCO groups searched
```

Conclusion

The BUSCO results show that 98.8% of the expected universal single-copy orthologs were found to be complete in the predicted protein sequences (97.9% single-copy, 0.9% duplicated), while only 0.4% were fragmented and 0.8% were missing.

This high completeness score indicates that the genome assembly and gene annotation for F. oxysporum Fov891A are of high quality and are suitable for downstream analyses, including functional annotation, effector prediction, and comparative genomics.

IV. BUSCO Result Visualization

In this step, we use the BUSCO output to visualize the completeness of the predicted protein sequences. A pie chart is generated in R to provide a more intuitive and easy-to-read summary of the results.

```
library(ggplot2)
library(dplyr)

# BUSCO data
busco <- data.frame(
  Category = c("Complete and single-copy", "Complete and duplicated", "Fragmented", "Missing"),
  Count = c(3738, 34, 16, 29)
)

# Custom color
custom_colors <- c(
  "Complete and duplicated"  = "#E76F51",
  "Complete and single-copy" = "#A9BBA9",
  "Fragmented"               = "#2A9D8F",
  "Missing"                  = "#9D4EDD"
```

```r
)

# Plot
ggplot(busco, aes(x = "", y = Count, fill = Category)) +
  geom_col(width = 1, color = "white") +
  coord_polar(theta = "y") +
  scale_fill_manual(values = custom_colors) +
  theme_void() +
  theme(
    legend.position = "right",
    legend.title = element_blank(),
    legend.text = element_text(size = 10)
  )
```

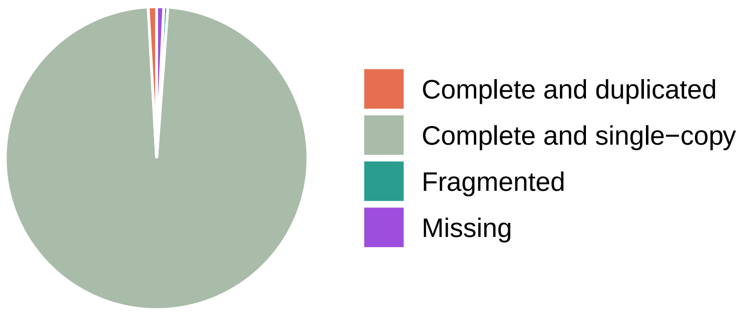


Figure 1: BUSCO Protein Completeness (Fov891A)

```r
# Generate a summary table
library(ggplot2)
library(dplyr)
library(knitr)

# Add percentage column
busco$Percentage <- round(busco$Count / sum(busco$Count) * 100, 1)

# Display the table
kable(busco, caption = "Summary of BUSCO Result Counts and Percentages")
```

Table 1: Summary of BUSCO Result Counts and Percentages

Category	Count	Percentage
Complete and single-copy	3738	97.9
Complete and duplicated	34	0.9
Fragmented	16	0.4
Missing	29	0.8

Chapter 2: Genome Quality and Annotation Validation

I. Literature Reference

Otero et al. (2024) – *Evaluation of the Genetic Diversity, Haplotype, and Virulence of Fusarium oxysporum* f. sp. *vasinfectum Field Isolates from Alabama.*
Doi: 10.1094/PHYTO-11-23-0438-R

This study revealed that the race 4 isolate 89-1A carries a set of small accessory chromosomes, including those harboring lactamase-related genes. These accessory chromosomes are postulated to contribute to the virulence of *Fusarium oxysporum* f. sp. *vasinfectum* on cotton, suggesting a potential role of horizontally transferred elements in pathogenicity.

II. Conclusion from the reference

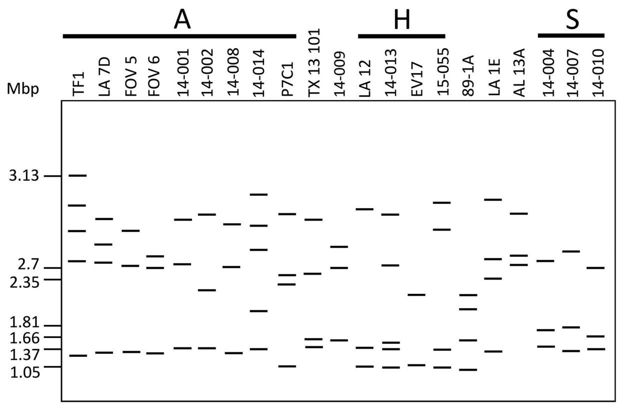


Figure 2. Small chromosome diversity in 21 Fusarium oxysporum f. sp. vasinfectum (Fov) field isolates assessed by pulsed-field gel electrophoresis.(Otero et al. 2024)

Conclusion.

The race 4 isolate **89-1A** shows distinct small chromosome bands compared to other isolates. These unique chromosomes are likely accessory chromosomes and may contain virulence-associated genes, such as lactamases, contributing to increased pathogenicity on cotton.

III. Localizing the Genes of Interest from a Specific Accessory Chromosome

Table 2: Location of the potential cotton-specific gene cluster (Otero et al. 2024)

Domain, (Sense)	89-1A Protein	Length	CA-12 Locus
Cupin, signal peptide (-)	FUN_000082, VEDA_05193	1,330 bp, 371 AA	1-1,330
Glutathione S-transferase (-)	FUN_000109, VdG1_00948	755 bp, 233 AA	1,975-2,729
Uncharacterized protein (-)	FUN_000146	447 bp, 148 AA	4,978-5,424
GMC oxidoreductase, signal peptide (-)	FUN_000208, VEDA_05195	2,118 bp, 621 AA	10,007-12,124
Uncharacterized protein (+)	FUN_000330	821 bp, 248 AA	19,825-20,644
SAM-dependent methyltransferase (-)	FUN_000376	1,109 bp, 211 AA	22,395-23,503
SAM-dependent methyltransferase (-)	FUN_000387	1,392 bp, 225 AA	23,531-24,922
FMN-dependent dehydrogenase (+)	FUN_000469	998 bp, 278 AA	28,966-29,963
zinc-binding alcohol dehydrogenase (-)	FUN_000504	309 bp, 102 AA	31,458-31,766
Alpha fold: possible integral membrane protein (+)	FUN_000631	375 bp, 101 AA	42,479-42,853
Alpha fold: possible integral membrane protein (+)	FUN_000642	746 bp, 230 AA	42,936-43,681
SAM-dependent methyltransferase (-)	FUN_000920	848 bp, 226 AA	74,477-75,324
Uncharacterized protein, signal peptide (-)	FUN_000947	848 bp, 135 AA	77,718-78,565
Uncharacterized protein (-)	FUN_000956	2,316 bp, 771 AA	79,888-82,203
Uncharacterized protein (-)	FUN_000985	219 bp, 72 AA	83,682-83,900
Alpha fold: F-box domain-containing protein (-)	FUN_001024	414 bp, 137 AA	88,219-88,632
Uncharacterized protein, secreted peptide (-)	FUN_001040	890 bp, 257 AA	90,190-91,079
SEST-like, SGNH hydrolase subfamily, plant suberin lipid hydrolase (+)	FUN_001051	4,914 bp, 1401 AA	91,772-96,685
AB hydrolase superfamily (+)	FUN_001144	857 bp, 245 AA	102,581-103,437
Uncharacterized protein (+)	FUN_001170	219 bp, 72 AA	105,029-108,112
Uncharacterized protein (+)	FUN_001185	2,316 bp, 771 AA	109,590-111,905
MFS superfamily (+)	FUN_001224	1,260 bp, 419 AA	113,926-115,185
short-chain dehydrogenase	FUN_001275	1,427 bp, 284 AA	119,041-120,467
Alpha fold: Nitrogen regulatory protein areA GATA-like domain-containing protein, LisH domain-containing protein (+)	FUN_001318	624 bp, 170 AA	123,450-124,073

Domain, (Sense)	89-1A Protein	Length	CA-12 Locus
Animal heme peroxidase-like superfamily (+)	FUN_001354	1,713 bp, 447 AA	127,416-129,128
Animal heme peroxidase-like superfamily, cytochrome P450 superfamily (+)	FUN_001373	2,078 bp, 568 AA	129,362-131,439
FAD-binding, GlcD superfamily	FUN_001409	1,719 bp, 572 AA	133,828-135,546
AB hydrolase, signal peptide (+)	FUN_001427	1,293 bp, 430 AA	136,208-137,499
alpha/beta hydrolase (+)	FUN_001452	1,663 bp, 452 AA	139,638-141,300
HeLo, HET prion, signal peptide (+)	FUN_001479	1,302 bp, 288 AA	142,100-143,401
Alpha/beta hydrolase superfamily (-)	FUN_001518	1,333 bp, 248 AA	147,119-148,451
Integrase, HTH Tc5 transposase DNA-binding protein (+)	FUN_019948	1,844 bp, 551 AA	152,051-153,894
Lactamase, signal peptide (-)	FUN_001580, VEDA_05199	1,333 bp, 416 AA	155,120-156,453
Zinc independent alcohol dehydrogenase (-)	FUN_001597, VEDA_05198	1,088 bp, 678 AA	157,732-158,820
Short-chain dehydrogenase (-)	FUN_001610, VEDA_05197	1,975 bp, 346 AA	158,821-160,796
MFS aflatoxin efflux pump (+)	FUN_001624, VEDA_05198	3,227 bp, 570 AA	160,937-164,164
GAL4-like zinc DNA-binding, sterol uptake (+)	FUN_001654	1,175 bp, 364 AA	164,353-165,528
SNU13 (-)	FUN_001849	1,054 bp, 217 AA	189,259-190,313
Ankyrin repeats (-)	FUN_001881	449 bp, 149 AA	193,530-193,979
sulfatase, alkaline phosphatase (-)	FUN_001892	2,258 bp, 733 AA	195,037-197,296

In **Table 2**, we identified a cluster of genes located on a specific accessory chromosome of *Fusarium oxysporum* f. sp. *vasinfectum* strain CA-12. These genes are absent in most other *F. oxysporum* strains and show high similarity to a conserved region in *Verticillium dahliae* strain Vd991.

Among the listed genes, we focused on **eight candidates** of particular interest. This selection was based on the following rationale:

1. Seven of these genes were previously reported as homologs shared between *F. oxysporum* and *V. dahliae* Vd991.
2. Our current analysis identified an **additional homolog**, expanding the set of conserved genes to eight (Chen et al., 2018).
3. These genes include **cupin, GMC oxidoreductase, GST, FMN-dependent dehydrogenase**, and multiple **SAM-dependent methyltransferases**, all of which are potentially involved in **host-specific adaptation**.
4. The presence of secretion signals and domain predictions in several genes supports their possible role as **accessory virulence factors**.
5. The entire cluster resides on a region absent from core chromosomes, suggesting its horizontal origin and role in **cotton-specific pathogenicity**.

This accessory cluster may therefore serve as a valuable genomic signature for Fov race 4 and a candidate target for disease resistance breeding in cotton.

VI. Localizing the Genes of Interest from a Specific Accessory Chromosome

Based on the previous analysis, we selected **eight genes of interest** located on a putative accessory chromosome for further investigation. These genes were chosen due to their predicted functions, presence of signal peptides, and high similarity to virulence-related genes in *Verticillium dahliae*. For details, see **Table 3** below.

Table 3: Candidate Genes list from the Accessory Chromosome

Domain, (Sense)	89-1A Protein	Length	CA-12 Locus
Cupin, signal peptide (-)	FUN_000082, VEDA_05193	1,330 bp, 371 AA	1-1,330
Glutathione S-transferase (-)	FUN_000109, VdG1_00948	755 bp, 233 AA	1,975-2,729
GMC oxidoreductase, signal peptide (-)	FUN_000208, VEDA_05195	2,118 bp, 621 AA	10,007-12,124
Lactamase, signal peptide (-)	FUN_001580, VEDA_05199	1,333 bp, 416 AA	155,120-156,453
Zinc independent alcohol dehydrogenase (-)	FUN_001597, VEDA_05198	1,088 bp, 678 AA	157,732-158,820
Short-chain dehydrogenase (-)	FUN_001610, VEDA_05197	1,975 bp, 346 AA	158,821-160,796
MFS aflatoxin efflux pump (+)	FUN_001624, VEDA_05198	3,227 bp, 570 AA	160,937-164,164
GAL4-like zinc DNA-binding, sterol uptake (+)	FUN_001654	1,175 bp, 364 AA	164,353-165,528

Chapter 3: Localization of Genes of Interest

In this section, we designed qPCR primers for 8 selected genes to analyze their expression patterns. This part presents the workflow and steps used to evaluate their amplification efficiency.

I. Efficiency test of the 8 selected genes

Summary of qPCR Primer Efficiency for 8 Genes

```r
library(readr)
library(dplyr)
library(tibble)
library(knitr)

# Read and clean data
df1 <- read_csv("test one--qPCR primer efficiency.csv") %>%
  select(-`...10`) %>%
  mutate(Source = "test one", `Avg Ct` = suppressWarnings(as.numeric(`Avg Ct`)))
```

```r
df2 <- read_csv("test two--qPCR primer efficiency.csv") %>%
  select(-`...10`) %>%
  mutate(Source = "test two", `Avg Ct` = suppressWarnings(as.numeric(`Avg Ct`)))

# Efficiency calculator
calculate_efficiency <- function(df, gene_list, source_label) {
  results <- lapply(gene_list, function(gene) {
    df_gene <- df %>% filter(`Gene name` == gene)
    if (nrow(df_gene) >= 3) {
      fit <- lm(`Avg Ct` ~ `Log (sample quantity)`, data = df_gene)
      slope <- coef(fit)[2]
      r2 <- summary(fit)$r.squared
      efficiency <- (10^(-1 / slope) - 1) * 100
      tibble(
        Gene = gene,
        Source = source_label,
        Slope = round(slope, 3),
        R_squared = round(r2, 4),
        Efficiency_percent = round(efficiency, 2)
      )
    } else {
      tibble(Gene = gene, Source = source_label,
             Slope = NA, R_squared = NA, Efficiency_percent = NA)
    }
  })
  bind_rows(results)
}

# Target genes
genes_one <- c("0082", "0109", "0208", "1580", "1654")
genes_two <- c("1597", "1610", "1624")

# Run and combine
table_one <- calculate_efficiency(df1, genes_one, "test one")
table_two <- calculate_efficiency(df2, genes_two, "test two")

efficiency_table <- bind_rows(table_one, table_two)

# Output as table
kable(efficiency_table, caption = "Efficiency Summary for 8 Selected Genes")
```

Table 4: Efficiency Summary for 8 Selected Genes

Gene	Source	Slope	R_squared	Efficiency_percent
0082	test one	-3.218	0.9772	104.53
0109	test one	-3.419	0.9677	96.10
0208	test one	-3.423	0.9610	95.95
1580	test one	-3.331	0.9603	99.62
1654	test one	-3.371	0.9687	97.99
1597	test two	-3.509	0.9887	92.74
1610	test two	-3.542	0.9509	91.57
1624	test two	-3.490	0.9775	93.43

II. Visualization of qPCR Primer Efficiency for 8 Genes

```r
# Read data from relative paths
df1 <- read_csv("test one--qPCR primer efficiency.csv") %>%
  mutate(Source = "test one",
         `Avg Ct` = suppressWarnings(as.numeric(`Avg Ct`)))

df2 <- read_csv("test two--qPCR primer efficiency.csv") %>%
  mutate(Source = "test two",
         `Avg Ct` = suppressWarnings(as.numeric(`Avg Ct`)))

# Combine data
df_all <- bind_rows(df1, df2)

# Define plotting function
plot_gene <- function(df, gene_name) {
  df_gene <- df %>% filter(`Gene name` == gene_name)

  if (nrow(df_gene) < 2) return(ggplot() + labs(caption = paste("Gene", gene_name, "(insufficient data)

  fit <- lm(`Avg Ct` ~ `Log (sample quantity)`, data = df_gene)
  slope <- coef(fit)[2]
  r2 <- summary(fit)$r.squared
  efficiency <- (10^(-1 / slope) - 1) * 100

  ggplot(df_gene, aes(x = `Log (sample quantity)`, y = `Avg Ct`)) +
    geom_point(size = 3, color = "steelblue") +
    geom_smooth(method = "lm", se = FALSE, linetype = "dashed", color = "firebrick") +
    annotate("text", x = min(df_gene$`Log (sample quantity)`), y = max(df_gene$`Avg Ct`) - 1,
             label = paste0("Slope = ", round(slope, 3),
                            "\nR² = ", round(r2, 3),
                            "\nEfficiency = ", round(efficiency, 2), "%"),
             hjust = 0, size = 4) +
    labs(caption = paste("Gene", gene_name),
         x = "Log10(Sample Quantity)",
         y = "Average Ct") +
    theme_minimal(base_size = 12) +
    theme(plot.caption = element_text(hjust = 0.5, face = "bold"))
}
```

```r
# Define genes from each source
genes_one <- c("0082", "0109", "0208", "1580", "1654")
genes_two <- c("1597", "1610", "1624")

# Generate plot list
plots <- list(
  `0082` = plot_gene(df1, "0082"),
  `0109` = plot_gene(df1, "0109"),
  `0208` = plot_gene(df1, "0208"),
  `1580` = plot_gene(df1, "1580"),
  `1654` = plot_gene(df1, "1654"),
  `1597` = plot_gene(df2, "1597"),
  `1610` = plot_gene(df2, "1610"),
  `1624` = plot_gene(df2, "1624")
)
```

```r
library(ggpubr)
annotate_figure(
  ggarrange(
    plots[["0082"]], plots[["0109"]],
    plots[["0208"]], plots[["1580"]],
    plots[["1654"]], plots[["1597"]],
    plots[["1610"]], plots[["1624"]],
    ncol = 2, nrow = 4,
    labels = NULL,
    align = "hv"
  ),
  bottom = text_grob("Figure: 8 Genes qPCR Primer Efficiency Test",
                     size = 16, hjust = 0.5)
)
```

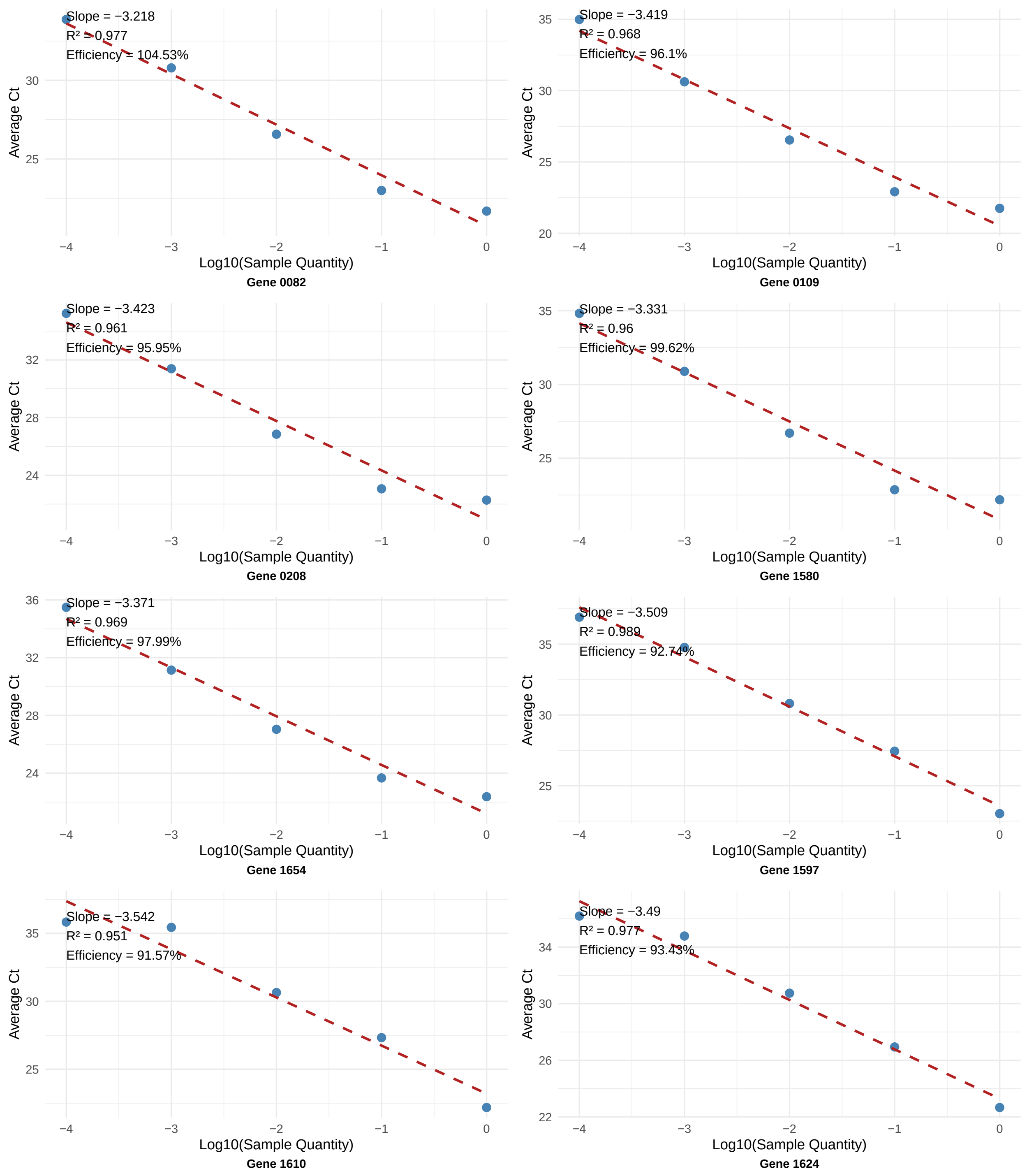


Figure: 8 Genes qPCR Primer Efficiency Test

Conclusion:

From the primer efficiency results, we observed that all eight gene primers had efficiencies ranging from 90% to 101%, indicating that they are suitable for subsequent RNA expression analysis.

In the next step, we used the Fusarium oxysporum strain 89-1A to infect cotton seedlings at the two-leaf stage. Root samples were collected at five time points: 0 hours, 15 hours, 1 day, 2 days, and 3 days post-inoculation.

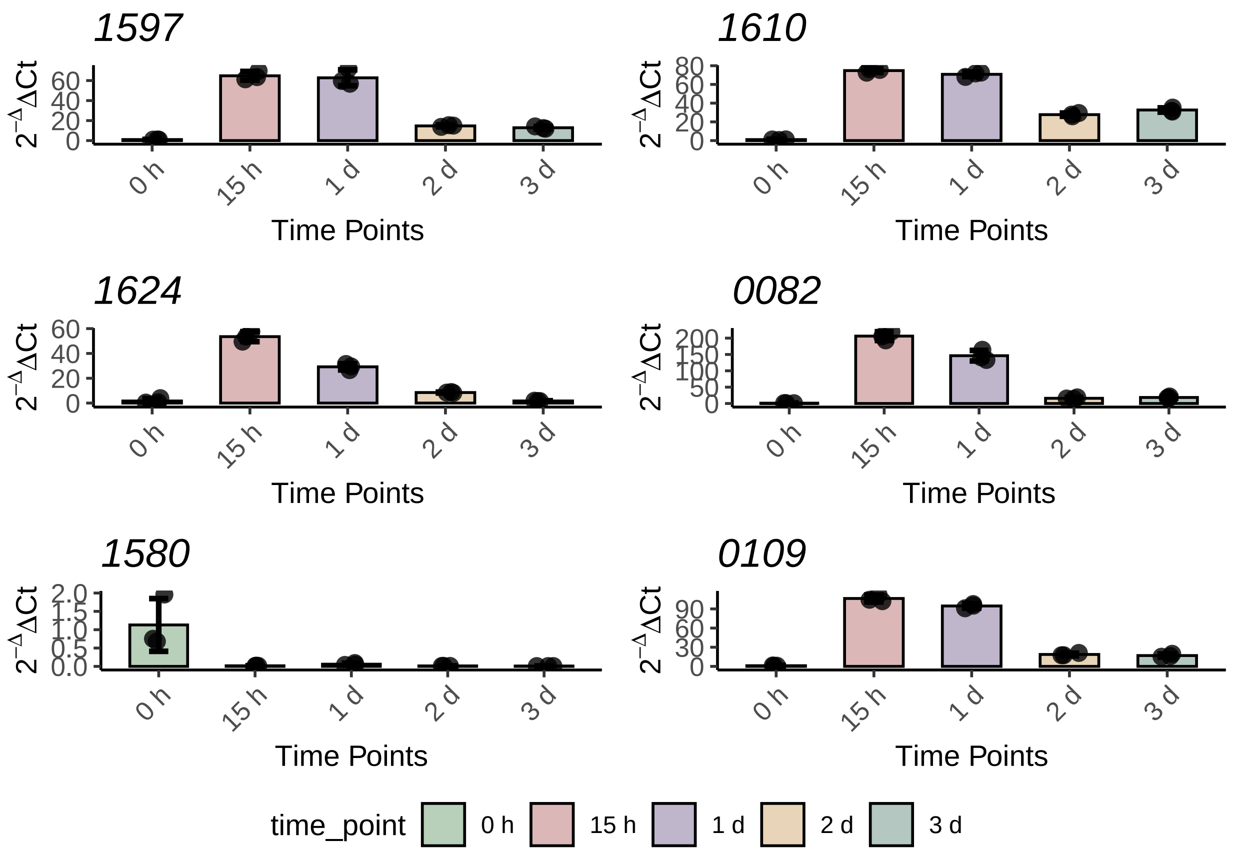
Total RNA was extracted using the E.Z.N.A.® Total RNA Kit, and gene expression analysis was performed on the collected samples.

III. qRT-PCR Analysis of 8 Selected Genes Across 5 Time Points

```r
library(ggplot2)
library(dplyr)
library(readr)
library(ggpubr)
```

```r
file_path <- "RNA expression data.csv"    ###read data
df_raw <- read_csv(file_path, col_names = FALSE)
plot_gene_expression <- function(df_raw, gene_id) {
  # Filter data for the selected gene
  df_gene <- df_raw %>%
    filter(X1 == gene_id) %>%
    mutate(
      time_point = trimws(gsub("-\\d+", "", X2)),  # Remove replicate ID (e.g., "-1", "-2")
      sample_Ct = as.numeric(X3),
      housekeeping_Ct = as.numeric(X4),
      delta_Ct = sample_Ct - housekeeping_Ct       # ΔCt = Sample Ct - Housekeeping Ct
    )

  # Calculate average ΔCt of 0h as baseline
  baseline <- df_gene %>%
    filter(time_point == "0 h") %>%
    summarise(mean_delta = mean(delta_Ct, na.rm = TRUE)) %>%
    pull()

  # Calculate ΔΔCt and relative expression (2^-ΔΔCt)
  df_gene <- df_gene %>%
    mutate(
      delta_delta_Ct = delta_Ct - baseline,
      relative_expression = 2^(-delta_delta_Ct)
    )

  # Compute mean and standard deviation for each time point
  summary_df <- df_gene %>%
    group_by(time_point) %>%
    summarise(
      mean_expression = mean(relative_expression),
      sd_expression = sd(relative_expression)
    )

  # Define time point order
  time_levels <- c("0 h", "15 h", "1 d", "2 d", "3 d")
  df_gene$time_point <- factor(df_gene$time_point, levels = time_levels)
  summary_df$time_point <- factor(summary_df$time_point, levels = time_levels)

  # Morandi-style color palette
  morandi_colors <- c(
    "0 h"  = "#A7C3A8",
    "15 h" = "#D4A5A5",
    "1 d"  = "#B0A4C0",
    "2 d"  = "#E3CBA8",
    "3 d"  = "#A2B9B2"
  )

  # Create the expression plot
  p <- ggplot() +
    geom_col(data = summary_df, aes(x = time_point, y = mean_expression, fill = time_point),
             color = "black", alpha = 0.8, width = 0.6) +
    scale_fill_manual(values = morandi_colors) +


    # Add error bars
    geom_errorbar(data = summary_df, aes(x = time_point,
                                         ymin = mean_expression - sd_expression,
                                         ymax = mean_expression + sd_expression),
                  width = 0.2, color = "black", size = 1) +

    # Plot individual technical replicates
    geom_jitter(data = df_gene, aes(x = time_point, y = relative_expression),
                color = "black", size = 2.5, width = 0.1, alpha = 0.8) +

    # Add axis labels and title
    labs(
      title = bquote(italic(.(gene_id))),
      x = "Time Points",
      y = expression(2^-Delta*Delta*Ct)
    ) +

    # Clean visual theme
    theme_classic() +
    theme(
      axis.text.x = element_text(angle = 45, hjust = 1, size = 10),
      axis.text.y = element_text(size = 10),
      plot.title = element_text(size = 15, face = "bold"),
      axis.title.x = element_text(size = 11),
      axis.title.y = element_text(size = 11)
    )

  return(p)
}
```

```r
# Define gene list and create plots
gene_ids <- c("1597", "1610", "1624", "0082", "1580", "0109")
gene_plots <- lapply(gene_ids, function(id) plot_gene_expression(df_raw, id))

# Display all plots in 2 rows × 3 columns layout
ggarrange(
  plotlist = gene_plots,
  ncol = 2, nrow = 3,
  common.legend = TRUE,
  legend = "bottom"
)
```



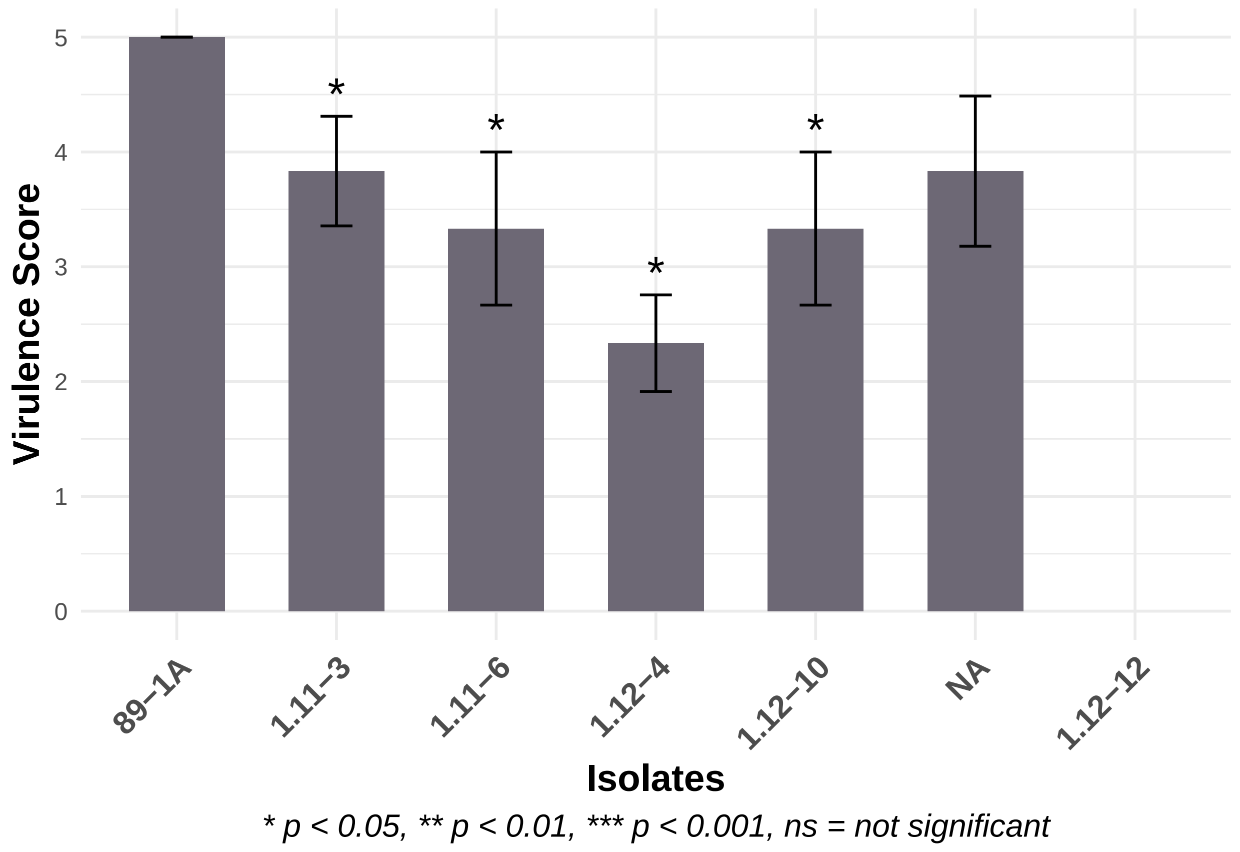
Chapter 4: Pathogenicity Assessment of Chromosome-Lost Isolates

As we've already localize 8 lactamase-related gene, we then use GFP tag labled the accessory chromosome that contained those 8 genes.(those 8 genes are in the same cluster of one accessory unique chromosome), through confocol-microscope we filtered those GFP tag was introduced into this chromosome, and two single-spore isolates(1.11, 1.12) carrying the GFP-tagged chromosome were obtained.

I. Hydroponic Test Tube Assay Confirms Virulence of GFP-Tagged Isolates A hydroponic test tube virulence assay demonstrated that the GFP-tagged isolates retained strong pathogenicity. Despite the insertion of the GFP marker, these isolates were still highly aggressive and caused severe disease symptoms on cotton seedlings, comparable to the wild-type strain.

```
library(ggplot2)
library(dplyr)
library(tidyr)
library(readxl)
library(ggpubr)

# Read the CSV file using a relative path from your project root
```

```r
df <- read.csv("gfp-tag isolates virulence test.csv")

# Rename the first column to 'variety' if needed
colnames(df)[1] <- "variety"

# Reshape the data into long format for ggplot compatibility
df_long <- df %>%
  pivot_longer(cols = -variety, names_to = "replicate", values_to = "score")

# Set factor levels to ensure plotting order
df_long$variety <- factor(df_long$variety, levels = c("89-1A", "1.11-3", "1.11-6", "1.12-4", "1.12-10",

# Summarize mean score and standard error by variety
df_summary <- df_long %>%
  group_by(variety) %>%
  summarise(
    mean_score = mean(score, na.rm = TRUE),
    se = sd(score, na.rm = TRUE) / sqrt(n())
  )

# Conduct pairwise t-tests comparing each strain with the control (89-1A)
control_group <- df_long %>% filter(variety == "89-1A")
comparison_varieties <- levels(df_long$variety)[-1]

# Initialize significance results dataframe
sig_results <- data.frame(variety = character(), p_value = numeric())

# Loop over varieties and perform t-tests
for (variety in comparison_varieties) {
  test_group <- df_long %>% filter(variety == variety)
  t_test_result <- t.test(control_group$score, test_group$score, var.equal = TRUE)
  sig_results <- rbind(sig_results, data.frame(variety = variety, p_value = t_test_result$p.value))
}

# Assign significance labels based on p-value
sig_results$label <- ifelse(sig_results$p_value < 0.001, "***",
                            ifelse(sig_results$p_value < 0.01, "**",
                                   ifelse(sig_results$p_value < 0.05, "*", "ns")))

# Merge with summary data to position labels above bars
sig_results <- sig_results %>%
  left_join(df_summary, by = "variety") %>%
  mutate(y_position = mean_score + se + 0.2)

# Create bar plot with error bars and significance annotations
ggplot(df_summary, aes(x = variety, y = mean_score)) +
  geom_bar(stat = "identity", fill = "#6D6875", width = 0.6) +
  geom_errorbar(aes(ymin = mean_score - se, ymax = mean_score + se),
                width = 0.2, color = "black") +
  labs(x = "Isolates", y = "Virulence Score") +
  theme_minimal() +
  theme(
    axis.text.x = element_text(angle = 45, hjust = 1, face = "bold", size = 12),
    axis.title = element_text(face = "bold", size = 14),
    plot.caption = element_text(size = 12, face = "italic", hjust = 0.5)
  ) +
  geom_text(data = sig_results, aes(x = variety, y = y_position, label = label),
            size = 6, color = "black") +
  labs(caption = "* p < 0.05, ** p < 0.01, *** p < 0.001, ns = not significant")
```

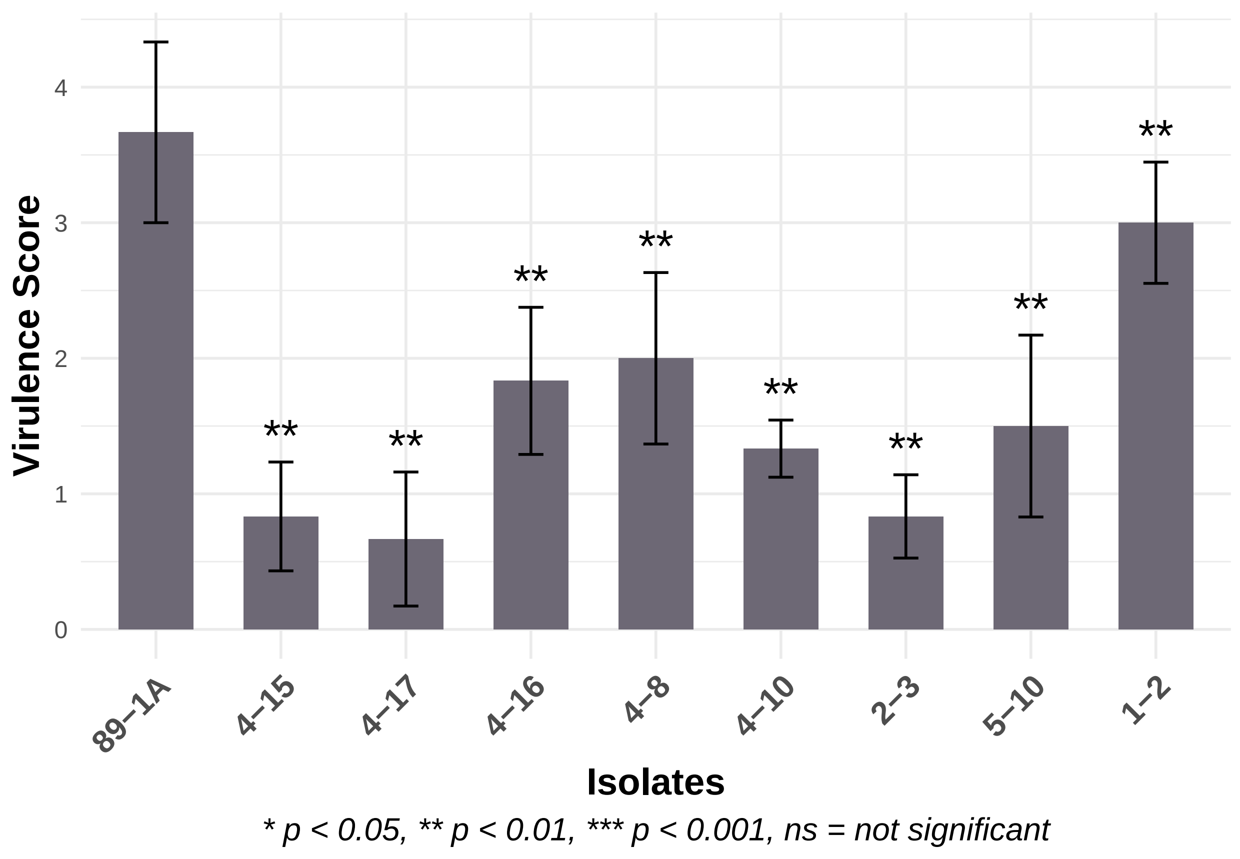


II. Virulence Test of GFP-Tagged Isolates

Using one of the GFP-tagged isolates, we performed flow cytometry screening and successfully isolated 44 conidia lacking GFP fluorescence, indicating they may have spontaneously lost the target chromosome. Subsequent virulence assays revealed isolates with a marked reduction in virulence.

```
library(ggplot2)
library(dplyr)
library(tidyr)
library(ggpubr)

# Read CSV using relative path from project root
df <- read.csv("3-17 chro-lost virulence test.csv")

# Rename the first column to 'variety' (if unnamed)
colnames(df)[1] <- "variety"
```

```r
# Convert wide format to long format
df_long <- df %>%
  pivot_longer(cols = -variety, names_to = "replicate", values_to = "score")

# Set factor levels for ordering
df_long$variety <- factor(df_long$variety, levels = unique(df_long$variety))

# Calculate mean and standard error
df_summary <- df_long %>%
  group_by(variety) %>%
  summarise(
    mean_score = mean(score, na.rm = TRUE),
    se = sd(score, na.rm = TRUE) / sqrt(n())
  )

# Prepare control and test varieties for t-tests
control_group <- df_long %>% filter(variety == levels(df_long$variety)[1])  # Use first variety as cont
comparison_varieties <- levels(df_long$variety)[-1]

# Run t-tests and collect significance
sig_results <- data.frame(variety = character(), p_value = numeric())

for (variety in comparison_varieties) {
  test_group <- df_long %>% filter(variety == variety)
  t_test_result <- t.test(control_group$score, test_group$score, var.equal = TRUE)
  sig_results <- rbind(sig_results, data.frame(variety = variety, p_value = t_test_result$p.value))
}

# Add significance label
sig_results$label <- ifelse(sig_results$p_value < 0.001, "***",
                            ifelse(sig_results$p_value < 0.01, "**",
                                   ifelse(sig_results$p_value < 0.05, "*", "ns")))

# Merge position info for annotations
sig_results <- sig_results %>%
  left_join(df_summary, by = "variety") %>%
  mutate(y_position = mean_score + se + 0.2)

# Draw bar plot with error bars and significance labels
ggplot(df_summary, aes(x = variety, y = mean_score)) +
  geom_bar(stat = "identity", fill = "#6D6875", width = 0.6) +
  geom_errorbar(aes(ymin = mean_score - se, ymax = mean_score + se),
                width = 0.2, color = "black") +
  labs(x = "Isolates", y = "Virulence Score") +
  theme_minimal() +
  theme(
    axis.text.x = element_text(angle = 45, hjust = 1, face = "bold", size = 12),
    axis.title = element_text(face = "bold", size = 14),
    plot.caption = element_text(size = 12, face = "italic", hjust = 0.5)
  ) +
  geom_text(data = sig_results, aes(x = variety, y = y_position, label = label),
            size = 6, color = "black") +
  labs(caption = "* p < 0.05, ** p < 0.01, *** p < 0.001, ns = not significant")
```



Virulence Score
0
1
2
3
4
**
89–1A
4–15
4–17
4–16
4–8
4–10
2–3
5–10
1–2
Isolates
* p < 0.05, ** p < 0.01, *** p < 0.001, ns = not significant