

Article

ZmC2GnT Positively Regulates Maize Seed Rot Resistance Against *Fusarium verticillioides*

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Abstract: *Fusarium verticillioides* can systematically infect maize through seeds, triggering stalk rot and ear rot at a later stage, thus resulting in yield loss and quality decline. Seeds carrying *F. verticillioides* are unsuitable for storage and pose a serious threat to human and animal health due to the toxins released by the fungus. Previously, the candidate gene *ZmC2GnT* was identified, using linkage and association analysis, as potentially implicated in maize seed resistance to *F. verticillioides*; however, its disease resistance mechanism remained unknown. Our current study revealed that *ZmC2GnT* codes an N-acetylglucosaminyltransferase, using sequence structure and evolutionary analysis. The candidate gene association analysis revealed multiple SNPs located in the UTRs and introns of *ZmC2GnT*. Cloning and comparing *ZmC2GnT* showed variations in the promoter and CDS of resistant and susceptible materials. The promoter of *ZmC2GnT* in the resistant parent contains one extra cis-element ABRE associated with the ABA signal, compared to the susceptible parent. Moreover, the amino acid sequence of *ZmC2GnT* in the resistant parent matches that of B73, but the susceptible parent contains ten amino acid alterations. The resistant material BT-1 and the susceptible material N6 were used as parents to observe the expression level of the *ZmC2GnT*. The results revealed that the expression of *ZmC2GnT* in disease-resistant maize seeds was significantly up-regulated after infection with *F. verticillioides*. After treatment with *F. verticillioides* or ABA, the expression activity of the *ZmC2GnT* promoter increased significantly in the resistant material, but no discernible difference was detected in the susceptible material. When *ZmC2GnT* from resistant and susceptible materials was overexpressed in *Arabidopsis thaliana*, the seeds' resistance to *F. verticillioides* increased, although there was no significant difference between the two types of overexpressed plants. Our study revealed that *ZmC2GnT* could participate in the immune process of plants against pathogenic fungus. *ZmC2GnT* plays a significant role in regulating the disease-resistance process of maize seeds, laying the foundation for future research into the regulatory mechanism and the development of new disease-resistant maize varieties.

Keywords: maize; *Fusarium verticillioides*; N-acetylglucosaminyltransferase; *ZmC2GnT*; cis-element ABRE



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1. Introduction

Maize has emerged as one of the most significant cereals in the world due to its high yield and tremendous demand for food, feed, and industrial applications. Maize is exposed to various biotic stresses throughout its life cycle, substantially affecting seed yield and quality. The incidence of soil-borne diseases in maize in China's Huang-Huai region has

steadily grown. *Fusarium verticillioides* (formerly *Fusarium moniliforme*) is a common soil-borne fungus that can spread to maize seeds and cause systemic infection [1]. Previous research has shown that infected seeds are one of the sources of root and stalk infections [2]. Moreover, fungal diseases can spread from infected seeds planted in the field to developing grains and then mature plants, leading to various root rots, stalk rots, ear rots, and seed rots [3–5].

F. verticillioides infection can produce intermediate metabolic toxins such as fumonisins, moniliformins, trichothecenes, fusaric acid, and isoleucyl jasmonic acid. Fumonisins, for example, can cause various diseases when consumed by animals and have been associated with human carcinogenesis, neural tube defects, and plant diseases, posing a serious threat to both human and livestock health. At present, the World Health Organization International Agency for Research on Cancer has designated it as a Group 2B carcinogen [6–8].

Disease-resistant breeding has emerged as an effective ecological safety strategy and a practical approach to enhance soil-borne disease resistance. However, only a limited number of resistance genotypes are currently applicable in agricultural production because of the highly complex regulatory mechanisms and control by multiple genes, which are heavily influenced by the environment [9–12]. Previous studies have used a combination of genome-wide association studies (GWASs) and quantitative trait loci (QTL) to locate and validate loci of complex characteristics, overcoming the limitations of using either method alone [13–16]. It has now become a powerful research strategy for obtaining target genes. The current research on resistance to *F. verticillioides* mainly focuses on Fusarium ear rot (FER), and a series of QTLs and candidate genes related to ear rot resistance have been identified. Nevertheless, there are extremely few studies conducted on the genetic complexity and identification of potential genes associated with Fusarium seed rot (FSR) resistance. Previous research has primarily focused on field evaluations, which are time-consuming, labor-intensive, and influenced by numerous environmental factors [17–20]. Therefore, a biological detection method was used to determine the severity of infectious diseases in the laboratory [21–23]. In this study, fungal suspensions were incubated with healthy mature seeds to investigate the degree of pathogen-induced seed rot. This approach has been successfully applied to assess maize resistance to *F. verticillioides* and can be fully controlled in a lab setting to produce more accurate phenotypic data [24,25].

O-GlcNAc is a dynamic and reversible post-translational modification found on serine/threonine residues of proteins that regulate multiple cellular pathways, including those related to plant physiology and pathology [26]. In vivo, N-acetylglucosaminyltransferase (OGT) and N-acetylglucosaminidase (OGA) carry out O-GlcNAc glycosylation modification. OGT exists simultaneously in the nucleus and cytoplasm, and O-GlcNAc glycosylation of proteins has been reported in both locations [27]. In recent years, due to the association of O-GlcNAc glycosylation mediated by N-acetylglucosaminyltransferase with diseases such as neurodegenerative disorders, diabetes, and cancer, it has increasingly become a research focus in glycobiology. However, research on protein glycosylation modification-related genes, especially N-acetylglucosaminyltransferase, in plant disease resistance is limited.

Glycosyltransferases play a role in plant stress resistance. Salt induces the up-regulation of glycosyltransferases *TaUGT1* and *TaUGT2* in wheat [28]. The increased expression of the oligo-glycosyltransferase subunit gene *STT3a* in *Dunaliella salina* can improve salt adaptation and flagella regeneration [29]. Transforming the sucrose-1-fructosyltransferase 1-SST gene can improve drought tolerance in tobacco [30]. Uridine diphosphate glycosyltransferase (UGT) can glycosylate DON to DON-3-O-glucoside, reducing its toxicity [31]. N-acetylglucosaminyltransferase (GnT) is a significant regulator of salt tolerance traits in upland cotton [32]. In rice, β -1,6-N-acetylglucosaminyltransferase (C2GnT) FC116 regu-

lates rice cell wall polymers and helps the cell wall function as a barrier against invading pathogens [33].

Our previous study identified a major resistance QTL *qRsfv5* for maize seed rot on chromosome 5. The candidate gene *ZmC2GnT*, which encodes an N-acetylglucosaminyltransferase, contains nine closely related markers inside this interval [24]. In this study, it was discovered that *F. verticillioides* infection significantly increased the expression of *ZmC2GnT* in the parental line BT-1, confirming that *ZmC2GnT* responds to *F. verticillioides* infection of maize in the resistant parent. The GUS staining experiment revealed that the difference in *ZmC2GnT* expression between the resistant and susceptible parents is because of an additional critical ABA-related element, ABRE, on the promoter of the resistant parent BT-1. Consequently, our findings suggest that *ZmC2GnT* is involved in seed resistance to *F. verticillioides* and responds to ABA signal.

2. Materials and Methods

Table S1 includes the primer sequences used in all the experiments.

2.1. Plant Materials and Growth Conditions

For *F. verticillioides*, the inbred line BT-1 was used as the resistant parent, and N6 as the susceptible parent [34]. The association population consisted of 217 inbred lines. These materials consisted of a rich genetic variation in maize inbred lines, sourced from the representative P-group, TSPT, Lancaster, Reid, etc., as well as tropical and subtropical lines from CIMMYT. Phenotypic statistics and association analysis were performed by these materials' seeds inoculated with *F. verticillioides*. A study by Ju et al. provided detailed information [24].

Arabidopsis (*Arabidopsis thaliana*) overexpression plants were developed in the Columbia (Col-0) background. In this experiment, transgenic lines were obtained by impregnating the flower buds of *Arabidopsis*, screened by basta, and identified by cloning primers. Homozygous transgenic lines of T3 generation were used for analysis. They were grown in soil at 22 °C under a 16 h light/8 h dark photoperiod. Sterilized plump seeds were vernalized in the dark at 4 °C for 3 d and sown on plates. The GUS staining and RNA isolation assays employed 1/2 MS medium (Coolaber, Beijing, China). Plates were sealed and incubated for growth at 22 °C under a 16 h light/8 h dark photoperiod.

2.2. Plasmid Construction

Target genes were cloned into vector pCAMBIA3301 to generate *Arabidopsis* overexpression plants. For promoter–GUS constructs, the promoter sequences were cloned into the vector pCAMBIA1391.

2.3. Seed Inoculation

First, we prepared potato dextrose agar (PDA) by cutting 200 g of potatoes into small pieces, boiling them in water, and filtering them through eight layers of gauze. After filtration, we added 20 g glucose and 15 g agar powder to this solution and adjusted the volume by ddH₂O to 1 L. The preserved strains were activated on this PDA medium at 28 °C for mycelia maturation in the incubator. The mature mycelia were then inoculated in PDA liquid medium and incubated overnight at 200 rpm. The spore fluid obtained in a high concentration was first microscopically examined and sterilized. The concentration was then diluted to 1×10^5 per milliliter with ddH₂O.

Maize seeds were disinfected with 75% alcohol for 30 s and 3% sodium hypochlorite for 15 min, followed by 3–5 ddH₂O washes. Disinfected seeds were cleaned with ddH₂O after being steeped in spore fluid and ddH₂O for 36 h and then placed on Petri plates lined with filter paper. Ten maize seeds in each Petri dish or 100 *Arabidopsis* seeds were put in each

area of the four-zone Petri dish. The seeds were inoculated with water and *F. verticillioides* overnight, in the dark, and then transferred to a light incubator for cultivation [35,36].

2.4. Gene Expression Analysis

Total RNA was isolated, and the cDNA was synthesized as previously described [24]. Seeds were inoculated with *F. verticillioides* for 0, 6, 12, 24, 36, and 72 h before being analyzed for gene expression profiles. The expression of the EF1a gene was utilized as an internal control to normalize all data. Each experiment involved at least three independent biological replicates. Gene expression was determined through RT-qPCR assays, using a StepOne Plus Real Time PCR system. It was performed according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA).

2.5. GUS Staining

Tobacco seedlings were collected 20 days after germination, and treated with *F. verticillioides* and ABA for 3 h each before being rinsed three times with sterile water. The tobacco seedlings were then treated for another 1.5 days before being rinsed three times with sterile water.

The seedlings were incubated in a stain solution (50 mM Na₂HPO₄, 50 mM NaH₂PO₄, 0.1% Triton X-100, 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆·3H₂O, and 2 mM X-Gluc) at 37 °C for the specified duration. Following GUS staining, the seedlings were immersed in 75% ethanol to decolorize before being examined with a stereomicroscope.

3. Results

3.1. ZmC2GnT Encodes a Putative β -1,6-N-Acetylglucosaminyltransferase Protein

F. verticillioides is a highly toxic plant pathogen that can systematically infect maize through the seed route, causing maize FSR. FSR is a key trait that influences maize yield and quality. Previous research, combined with quantitative trait loci (QTL) mapping and genome-wide association study (GWAS) analysis, identified nine SNPs (Single-Nucleotide Polymorphisms) in the candidate gene GRMZM2G099255 [24]. No studies on this gene in maize have been published specifically to date.

The maizeGDB database contains information about the B73 reference gene. The gene structure of GRMZM2G099255 shows that it has ten exons and nine introns in maize (Figure 1A). The protein structure predicted by SMART suggests that ZmC2GnT has a Pfam domain, whose annotation indicates that this domain belongs to the glycosyltransferase family 14. It is a beta-1,6-N-acetylglucosaminyltransferase family consisting of I-branching enzyme and core-2 branching enzyme. In the SMART database, it explicitly pointed out that ZmC2GnT is a core-2/I-branching beta-1,6-N-acetylglucosaminyltransferase family protein (Figure 1B) [37–39]. BLAST searched homologous gene sequences in various species using the NCBI database, including *Sorghum bicolor*, *Arabidopsis thaliana*, *Triticum aestivum*, *Oryza sativa*, and *Zea mays*. MEGA 11 was used to analyze sequence alignment and construct a phylogeny tree (Figure 1C). This gene is also highly homologous to β -1,6-N-acetylglucosaminyltransferase found in maize and sorghum. Therefore, it was named ZmC2GnT for functional research. N-acetylglucosaminyltransferase is primarily involved in glycosylation in plants and has a significant role in plant growth and development, as well as response to biotic and abiotic stimuli [26,33]. ZmC2GnT demonstrates ubiquitous expression in almost all tissues sampled throughout the maize life cycle, including germinating seeds [40]. It is concluded that ZmC2GnT encodes a putative β -1, 6-N-acetylglucosaminyl transferase that may regulate maize seed immunity to *F. verticillioides*.

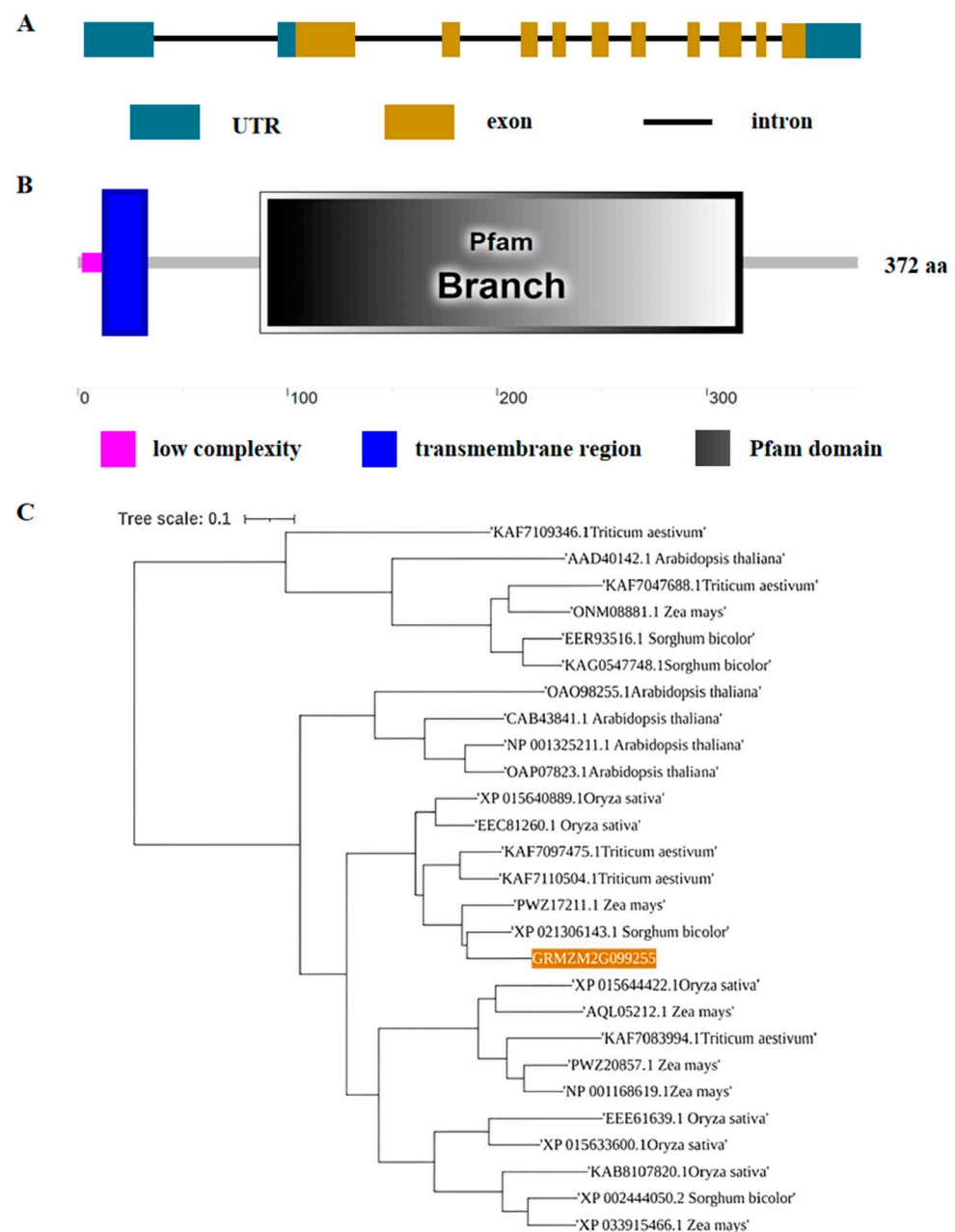


Figure 1. The structural and phylogenetic analysis of the candidate gene *ZmC2GnT*. (A) Gene structure of *ZmC2GnT* (GRMZM2G099255). The dark green boxes represent UTRs, the deep yellow boxes represent exons, and the black lines represent introns. (B) Cartoon of *ZmC2GnT* protein predicted by <http://smart.embl-heidelberg.de/> (accessed on 15 October 2024). The purple box represents low complexity, the blue box denotes the transmembrane region, and the black box is the Pfam domain. (C) Phylogeny tree of *ZmC2GnT* of maize and other related plant proteins. It was constructed using the MEGA 11 (<https://www.megasoftware.net/>, accessed on 15 October 2024). The *ZmC2GnT* homologous sequences from *Sorghum bicolor*, *Arabidopsis thaliana*, *Triticum aestivum*, *Oryza sativa*, and *Zea mays* were obtained by a BLAST search of the NCBI database.

3.2. *ZmC2GnT* Helps Seeds Resist *F. verticillioides*

The candidate gene association analysis revealed nine SNPs significantly associated with resistance to *F. verticillioides*, located in 5'UTR, 3'UTR, and introns of *ZmC2GnT* (Figure 2). The SNPs are 5_58174632, 5_58176692, 5_58176725, 5_58176831, 5_58177019, 5_58177074, 5_58177075, 5_58176782, 5_58165368, and 5_58178467. Among them, 5_58178467 is located in 5'UTR, 5_58174632 in 3'UTR, and the rest in introns. The

marker 5_58174632, which was most significantly associated with disease grade, was located on the 3'UTR, dividing the gene into two haplotypes, "CC" and "TT". The mean disease grade of inbred lines with "CC" haplotype was 3.89, significantly higher than that of "TT" haplotype (2.93). This shows that variations in *ZmC2GnT* expression or function in various maize lines may affect seed resistance, but the specific mechanism is unknown.

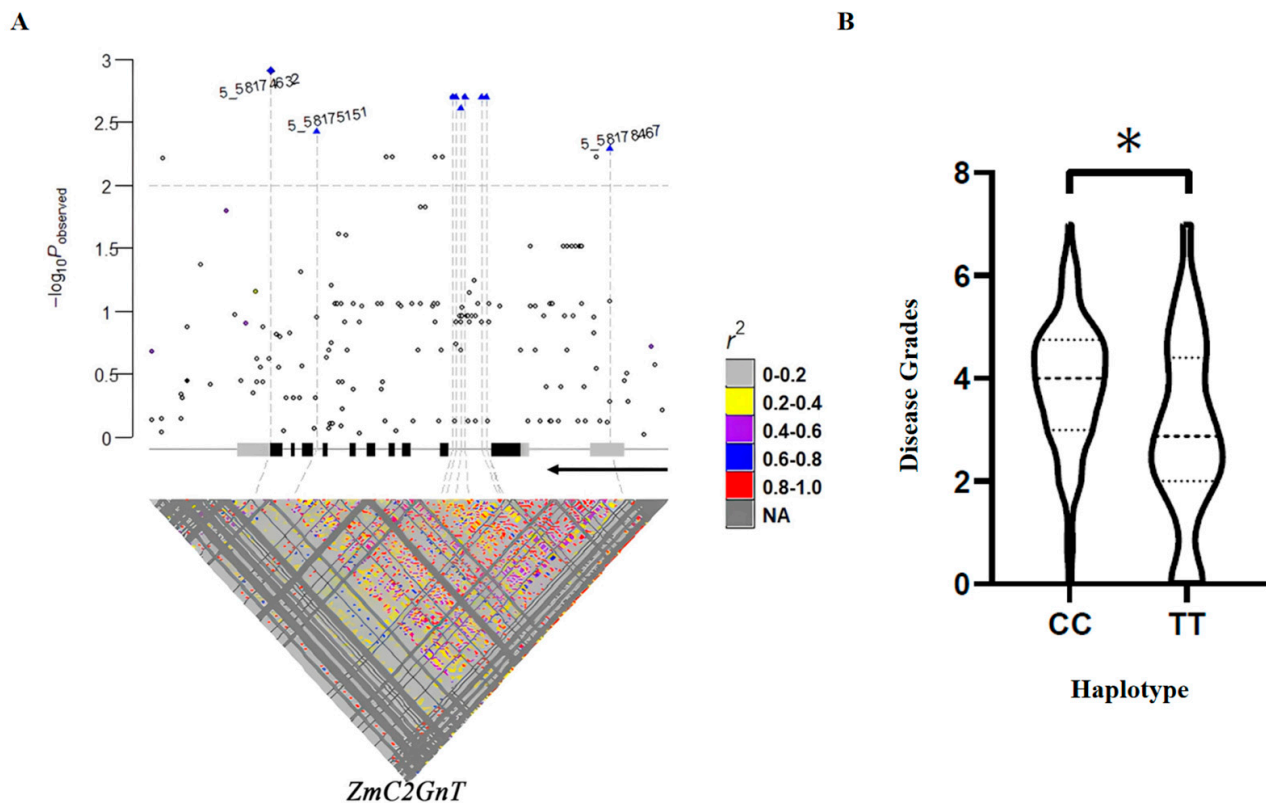


Figure 2. The association analysis of the candidate gene *ZmC2GnT*. (A) SNPs in the gene *ZmC2GnT* and its upstream 5 kb and downstream 1 kb were isolated from 217 lines using resequencing data, and association analysis was performed. The blue triangle represents SNPs significantly associated with resistance. The positions are 5_58174632, 5_58176692, 5_58176725, 5_58176831, 5_58177019, 5_58177074, 5_58177075, 5_58176782, 5_58165368, and 5_58178467. The black arrow represents the genetic direction. The inverted triangle reflects LD values. Gray, 0–0.2; yellow, 0.2–0.4; purple, 0.4–0.6; blue, 0.6–0.8; and red, 0.8–1.0. (B) Haplotypes analysis associated with disease grade. The mean disease grade of inbred lines has two haplotypes, "CC" and "TT". * $p < 0.05$.

The parental line BT-1 shows high resistance to *F. verticillioides*, while the parental line N6 is highly susceptible [34]. The two parental lines were used to clone the promoter and CDS sequences. Sequence alignment revealed variations in the promoter and CDS of the resistant and susceptible materials. The biparental promoters of *ZmC2GnT* differ in various ways (Supplementary Figure S1). The functional elements of the promoter region were evaluated on the Plant Care website, and it was discovered that it had one more cis-element, ABRE, associated with the ABA signal at −166 bp in BT-1 than N6 (Supplementary Figure S1 and Table S2). The CDS sequence of BT-1 is consistent with that of B73, and 13 base mutations occurred in the susceptible inbred line N6 (Supplementary Figure S2A). These 13 bases make a difference of ten amino acids in N6 (Supplementary Figure S2B).

3.3. The Expression of *ZmC2GnT* Increases in Response to *F. verticillioides* Infection

The results of the candidate gene association analysis suggested that differences in the expression of *ZmC2GnT* caused differences in the resistance of various inbred maize lines to *F. verticillioides*. BT-1 and N6 were used as the resistant material and the susceptible material, respectively [34]. The BT-1 and N6 seeds were inoculated with *F. verticillioides* to determine whether *ZmC2GnT* exhibited any difference in expression. The qRT-PCR assay was used to measure the expression level of *ZmC2GnT* at 0 h, 6 h, 12 h, 24 h, 36 h, and 72 h (Figure 3).

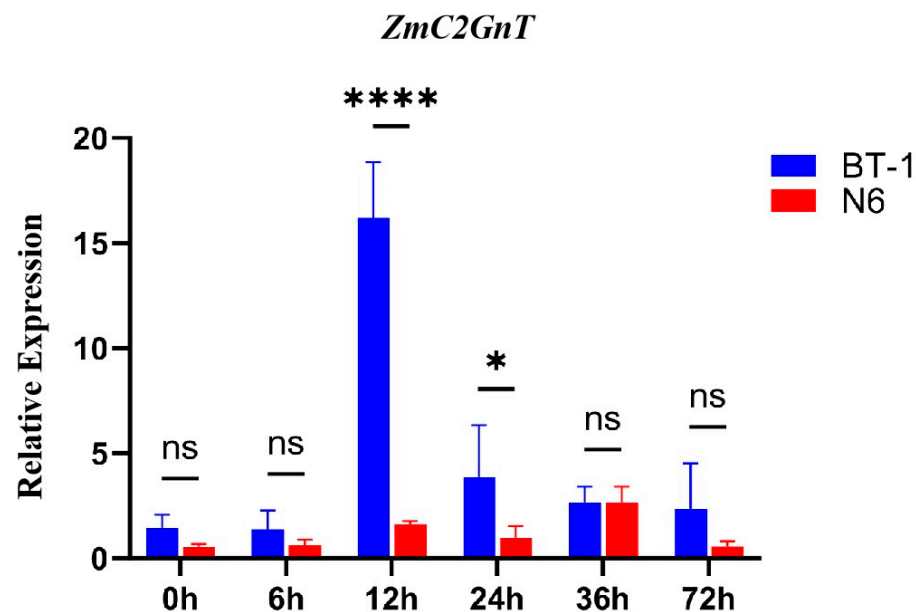


Figure 3. Expression analysis of *ZmC2GnT*. Expression analysis of *ZmC2GnT* in the seeds of BT-1 and N6 at different time points after treatment with *F. verticillioides* using RT-qPCR. Data are means $\pm$ SD from three biological replicates. * $p < 0.05$; **** $p < 0.0001$; ns, no significant difference.

ZmC2GnT expression levels were significantly different at 12 h and 24 h following *F. verticillioides* induction. They were significantly up-regulated in BT-1, reaching their highest level at 12 h. However, the expression levels of *ZmC2GnT* in N6 showed a relatively low and delayed up-regulation, with expression reaching the peak at 36 h, and there were no significant differences from BT-1. The results revealed that infection with *F. verticillioides* significantly up-regulated *ZmC2GnT* in the resistant maize seeds. Therefore, it was concluded that up-regulated *ZmC2GnT* promotes the seed resistance to *F. verticillioides*.

3.4. The Cis-Element on the *ZmC2GnT* Promoter Is Responsible for Resistance Against *F. verticillioides*

Comparing functional elements on the *ZmC2GnT* promoter from biparental materials, *ZmC2GnT* in BT-1 contains one extra cis-element ABRE associated with the ABA signal than N6 (Supplementary Figure S1 and Table S2). To verify whether the alteration in *ZmC2GnT* expression levels was due to the cis-element ABRE responding to ABA signals, the GUS gene was driven by the promoters of 35S, *ZmC2GnT*^{BT-1}, or *ZmC2GnT*^{N6}, and transiently transformed into *N. benthamiana* seedlings treated with CK (control check), FV (*F. verticillioides*), and ABA (Figure 4). The promoter-driven GUS expression of *ZmC2GnT*^{BT-1} increased when treated with *F. verticillioides*, but *ZmC2GnT*^{N6} showed no significant difference. When treated with ABA, the GUS staining results were similar to those observed with *F. verticillioides*. This indicated that *ZmC2GnT* can be induced to enhance resistance, and this cis-element ABRE is critical in regulating *ZmC2GnT* expression in response to

F. verticillioides inoculation. Therefore, the presence of the cis-element ABRE may affect the differential resistance in BT-1 and N6 against *F. verticillioides*.

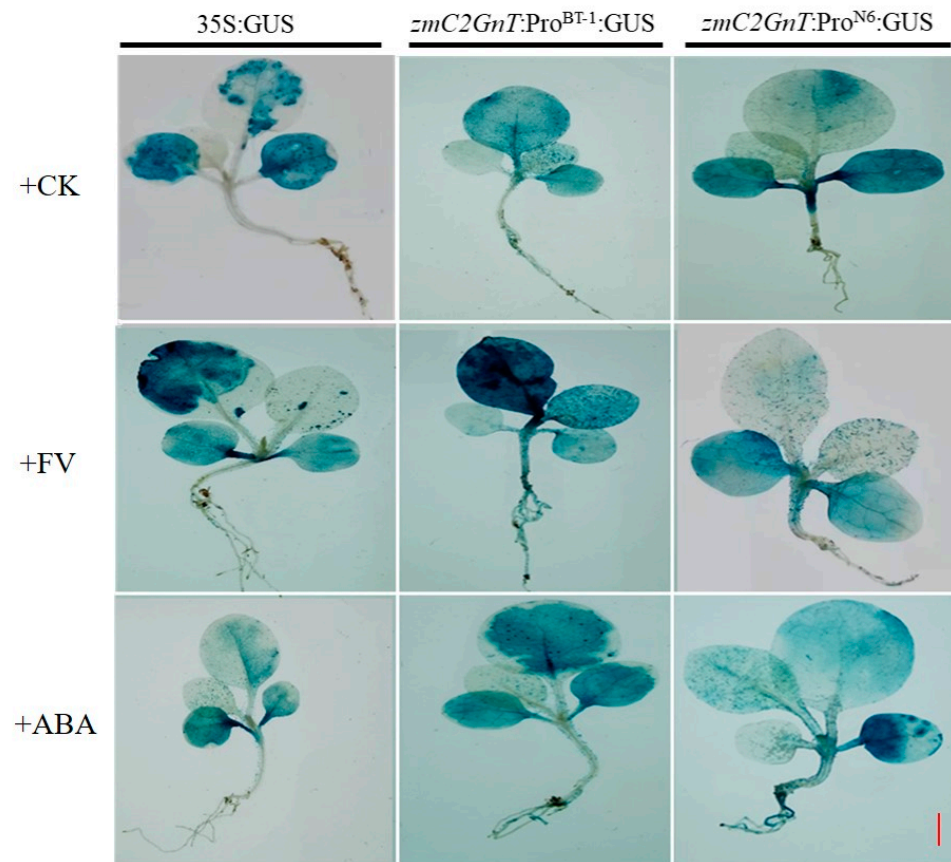


Figure 4. GUS promoter analysis of *ZmC2GnT* in BT-1 and N6 parental lines. The GUS gene, driven by promoters of *ZmC2GnT*^{BT-1} or *ZmC2GnT*^{N6}, was transiently transformed into *Nicotiana benthamiana* seedlings. GUS staining was used to determine the promoter activity when inoculated with water, *F. verticillioides*, and ABA, respectively. CK, control check; FV, *F. verticillioides*. Red scale = 2 mm.

3.5. *ZmC2GnT* Promotes Plant Immunity to *F. verticillioides*

Transgenic *ZmC2GnT*^{BT-1} and *ZmC2GnT*^{N6} overexpression plants were generated in *A. thaliana* ecotype Columbia-0 (Col-0) by using a constitutively active 35S promoter, and the potential immune function of *ZmC2GnT* against *F. verticillioides* was further explored. Due to the small size of *Arabidopsis* seeds, it is not easy to calculate the seed rot area or mycelium coverage area, and considering that seed rot directly affects the germination. The seed germination of *Arabidopsis* was used as the identification index [41–43]. The *Arabidopsis* wild-type (WT) seeds were inoculated with spore suspension of *F. verticillioides* at different concentrations to determine the optimal concentration for germination (Figure S3A). The results showed that when the concentration of spore suspension was 1×10^7 /mL, the seed activity and germination were inhibited (Figure S3B). Therefore, 1×10^7 /mL was established as the optimal concentration for inoculation.

The phenotypes of *ZmC2GnT*^{BT-1} and *ZmC2GnT*^{N6} overexpression lines in *Arabidopsis* inoculated with spore suspension were analyzed (Figure 5). WT seeds were treated with sterile water and *F. verticillioides* and used as controls. There was no significant difference in seed germination between the WT and transgenic overexpression lines after water treatment. Overexpression lines of *ZmC2GnT*^{BT-1} and *ZmC2GnT*^{N6} showed significantly higher germination numbers than WT seeds treated with *F. verticillioides* compared to the water treatment; however, there was no significant difference between the overexpression lines

of *ZmC2GnT*^{BT-1} and *ZmC2GnT*^{N6}. Thus, it is concluded that the germination numbers of overexpressed Arabidopsis seeds were higher than those of WT seeds after *F. verticillioides* treatment. Statistical analysis revealed no significant difference in resistance to *F. verticillioides* between overexpressed Arabidopsis seeds with *ZmC2GnT*^{BT-1} and *ZmC2GnT*^{N6}. It is preliminarily speculated that there is no difference in the potential immune function of *ZmC2GnT* to *F. verticillioides* in the disease resistance process between resistant and susceptible maize seeds.

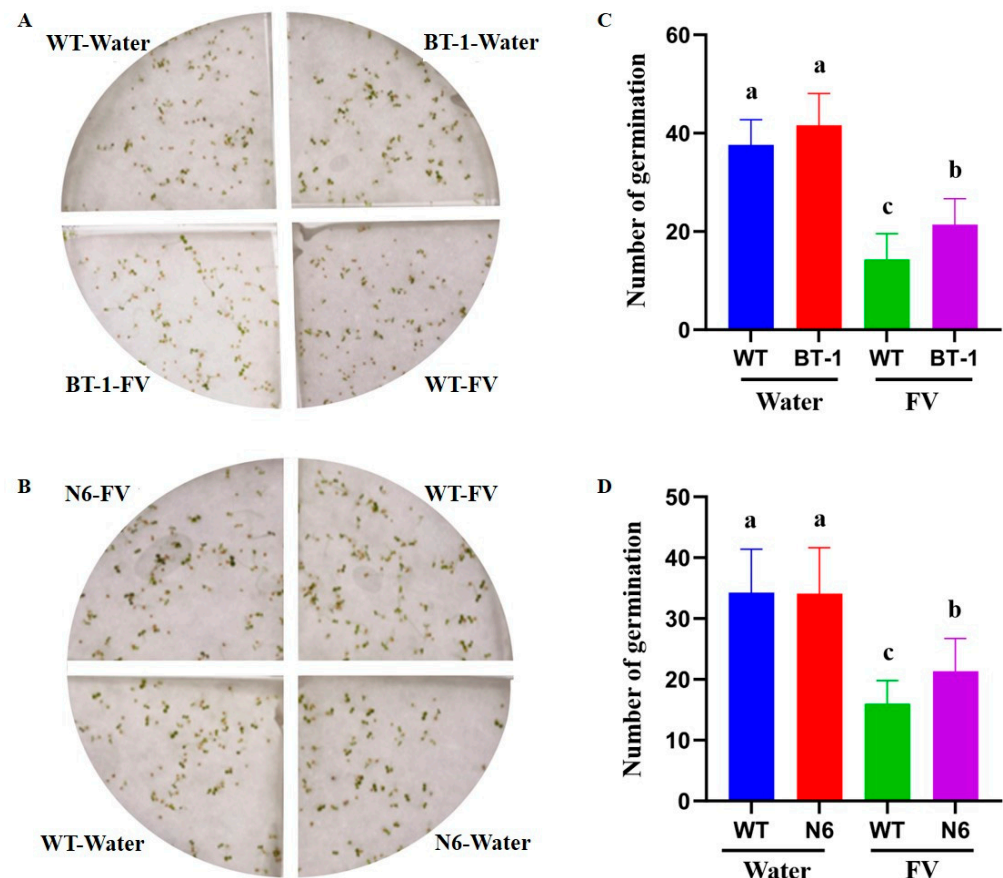


Figure 5. *ZmC2GnT* overexpressed transgenic lines of Arabidopsis showing increased resistance to *F. verticillioides*. (A) Arabidopsis wild-type (WT) and *ZmC2GnT*^{BT-1} overexpressed transgenic lines inoculated with water and *F. verticillioides* for ten days each. (B) Arabidopsis wild-type (WT) and *ZmC2GnT*^{N6} overexpressed transgenic lines inoculated with water and *F. verticillioides* for ten days each. (C,D) Germination rate of WT, *ZmC2GnT*^{BT-1}, and *ZmC2GnT*^{N6} after water and *F. verticillioides* treatment. The number of germinated seeds in (A,B) were counted. One hundred Arabidopsis seeds were placed initially in each area of the four-zone Petri dish. Data are presented as means $\pm$ SD, $n = 3$. One-way ANOVA was used for the statistical analysis. Significant differences are indicated with different letters. $p < 0.05$.

4. Discussion

Disease-resistant breeding in maize suppresses the spread of FSR at its source and is the most effective and environmentally friendly solution. However, the key to successfully utilizing this approach is to identify resistant sources that are effective and stable in various environments. It is critical for our overall understanding of how plants defend against pathogens. Our previous work revealed significant differences in the assessment of FSR resistance across 219 inbred lines [24]. Using a combination of QTL mapping and GWAS analysis, the candidate gene *ZmC2GnT* on chromosome 5 was identified as being significantly up-regulated by *F. verticillioides* induction. This gene encodes an N-

acetylglucosaminyltransferase. Rademacher and coworkers at Oxford University released an essay titled “Glycobiology” in 1988, which marked the formal birth of glycobiology [44]. Subsequent studies have primarily focused on animals and microorganisms, with little emphasis on plant glycobiology. As an essential part of glycobiology, glycosyltransferases play crucial roles in plant physiology, including hormone balancing, defense responses, detoxification reactions, secondary metabolism, and plant signal transduction. However, research on N-acetylglucosaminyltransferases in plant disease resistance has been exceedingly limited, with nearly no investigations on the gene *ZmC2GnT*. Therefore, our effort in this field is significant.

The candidate gene association analysis indicated that the UTR and intron of this gene include significant SNPs. The differences in *ZmG2nT* expression levels may be due to variances in the promoters of BT-1 and N6, leading to disparities in disease resistance phenotypes across the biparental lines. BT-1 and N6 maize seeds were infected with *F. verticillioides* for varying durations and studied the changes in *ZmG2nT* expression levels. Our study showed that, following pathogen induction, the expression level of *ZmC2GnT* was significantly up-regulated, peaking at 12 h and then progressively declining to its initial level in the resistant line. In contrast, the sensitive material had no significant variation in expression levels. It is concluded that the high expression of *ZmG2nT* in resistant lines contributes significantly to maize seed resistance. Compared to N6, the *ZmC2GnT* promoter contains one extra ABA-related element ABRE in BT-1. When *F. verticillioides* infects maize seedlings, corresponding transcription factors in the ABA signaling pathway bind to this cis-element, regulating the biotic stress response. Biparental promoters were used to drive the GUS (β -Glucuronidase) gene for transient expression in tobacco. Treatment with *F. verticillioides* or ABA significantly increased the expression activity of the *ZmC2GnT* promoter in the resistant material. However, no apparent difference was observed in the susceptible material, indicating that this cis-element is critical in *ZmC2GnT*'s response to *F. verticillioides* stress. To support this conclusion, a mutational experiment can be included. By substituting the N6 promoter sequence with that of BT-1 (and vice versa), it will help determine whether these changes affect *ZmC2GnT* expression levels and resistance to *F. verticillioides*. And then, the specific transcription factor of the ABA signaling pathway will be identified and evaluated if the binding of transcription factor and ABRE induced by *F. verticillioides* boost promoter activity.

Compared to the resistant line, ten amino acid mutations were observed in the protein *ZmC2GnT* in the susceptible line. Our findings revealed that overexpressing *ZmC2GnT* from resistant and susceptible materials in *Arabidopsis thaliana* increased seed resistance to *F. verticillioides*, although there was no significant difference between the *ZmC2GnT*^{BT-1} and *ZmC2GnT*^{N6} overexpressing lines. It was concluded that the natural mutations of *ZmC2GnT* in resistant and susceptible lines have little effect on its ability to participate in disease resistance control. Future research will investigate whether amino acid differences due to natural variation alter the protein activity of *ZmC2GnT*.

N-acetylglucosaminyltransferase occurs primarily in the Golgi apparatus, endoplasmic reticulum, nucleus, and cytoplasm. It synthesizes and processes asparagine-linked (N-) or serine/threonine-linked (O-) glycans. The evolutionary tree indicates that *ZmC2GnT* may belong to the N-acetylglucosaminyltransferase family. This family, which comprises the IGnT and C2GnT subfamilies, is involved in the synthesis of N-acetylgalactosamine from O-glycans (O-GlcNAc modification) and can be further modified into functional oligosaccharides. Under various stress conditions, the O-GlcNAc modification of nuclear and cytoplasmic proteins is blocked or reduced, making cells more susceptible to stress and decreasing the number of viable cells [45]. Under cellular stress, O-GlcNAc functions as a nutrient sensor, similar to protein phosphorylation, and is involved in stress-related

signal transduction pathways [46]. It is possible that *ZmC2GnT* serves a similar role and participates in seed defense against *F. verticillioides*.

In conclusion, *ZmG2nT* is highly expressed in maize seeds exposed to pathogens. It is hypothesized that *ZmG2nT* contributes to signal transduction, hormone regulation, or detoxification by glycosylating other proteins or small molecular compounds, thereby engaging in the stress response, maintaining normal physiological metabolism, and enhancing maize resistance. Further research is needed to determine *ZmG2nT*'s specific biological functions and regulatory mechanism underlying its involvement in maize seed disease resistance.

5. Conclusions

Glycosyltransferases have been demonstrated to play an important role in plant responses to biotic and abiotic stresses. However, research on N-acetylglucosaminyltransferases and their involvement in plant disease resistance remains limited. This study identified that *ZmC2GnT* is an N-acetylglucosaminyltransferase which plays a crucial role in maize seed resistance to *F. verticillioides*. The candidate gene association analysis showed that multiple SNPs are located in the UTRs and introns of *ZmC2GnT*. Gene expression analysis showed that *ZmC2GnT* was induced by *F. verticillioides* infection, and its expression significantly increased in the resistant parent. The GUS staining experiment revealed that the difference in *ZmC2GnT* expression between the resistant and susceptible parents is because of an additional critical ABA-related element, ABRE, on the promoter of the resistant parent, BT-1, compared to the susceptible parent, N6. Overexpressed *ZmC2GnT* in *Arabidopsis thaliana*, in the transgenic lines, showed more seed germination than WT under pathogen stress, indicating that *ZmC2GnT* increased seed resistance to *F. verticillioides*. In summary, the present study suggests that N-acetylglucosaminyltransferase could play an important role in the immune response of plants against pathogen. It also confirms that *ZmC2GnT* may be involved in the ABA signaling pathway that regulates disease-resistance process of maize seeds, laying the foundation for future research into the regulatory mechanism and the development of new disease-resistant maize varieties.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/agronomy15020461/s1>, Figure S1: Promoter region alignment of *ZmC2GnT* in BT-1 and N6; Figure S2: Sequence analysis of *ZmC2GnT*; Figure S3: Germination rate of wild-type *Arabidopsis thaliana* seeds at various inoculation concentrations; Table S1: List of primers used in this study; Table S2: Promoter region element analysis of *ZmC2GnT*.

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