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Cloning and Disease Resistance Analysis of the Maize *ZmBON3* Gene

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Abstract: (1) Corn is the most widely planted food crop, feed crop, and economic crop in the world, and plays an important role in agricultural production and national economy development. The *copine* gene, also known as the *BONZAI* gene, encodes a Ca^{2+}-dependent phospholipid membrane binding protein that is widely present in eukaryotes. It has been found that the copine protein is a negative regulator of disease resistance regulation and plays a key role in plants' disease resistance response. In this study, the Agrobacterium-tumefacien-mediated method was used to successfully obtain T2 generation *ZmBON3*-gene-overexpressing plants and gene-edited plants. Related phenotypes and molecular identification showed that the disease resistance of overexpression plants was significantly reduced, and the disease resistance of gene-edited plants was significantly increased, which verified that the *ZmBON3* gene was a negative regulatory gene. By detecting the physiological indexes related to defense, it was found that the content of H_2O_2 and the enzyme active water of CAT, POD, SOD, and PAL in *ZmBON3*-gene-edited plants was higher than those in the control plants and *ZmBON3*-gene-overexpressing plants, and the content of H_2O_2 and CAT, POD, and SOD in *ZmBON3*-gene-overexpressing plants was significantly higher than that in the control plants and *ZmBON3*-gene-overexpressing plants. The enzyme activity of PAL was the lowest. By detecting the expression of key genes of defense-related signaling pathways, it was found that *ZmBON3* may be involved in the related defense processes mediated by the R gene, SA pathway, JA pathway, and ABA pathway. In addition, *ZmBON3*-geneedited plants showed obvious dwarf phenomenon at the seedling stage, but this did not affect the ear length, axis diameter, ear row number, and grain color.

Keywords: maize; copine; *ZmBON3* gene; plant disease resistance; functional analysis

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1. Introduction

Maize (*Zea mays* L.) is a food crop with the largest planting area and yield in the world, as well as being an important feed crop and industrial raw material crop [1]. According to the data published by the National Bureau of Statistics of China, in 2019, the planting area of corn in China reached 41,284 thousand hectares, and the total output reached 260.77 million tons, ranking first in China's total grain [2]. The growth process of maize will be affected by a variety of adverse growth factors, among which disease is an important factor affecting maize yield. northern corn leaf blight (NCLB) is one of the most common maize diseases. Northern maize leaf blight is a common disease of maize. It is caused by Exserohilum turcicum, a filamentous fungus, which is the causative agent of NCLB in maize [3]. NCLB is commonly found in areas with temperate and tropical climates [4]. When the maize was infected, small water-drop-shaped spots appeared at first, and then expanded into large spots. The disease developed rapidly [5]. When it occurs, it can cause

about a 20% reduction in production, and when it breaks out, the reduction degree can rise to 46% [6].

As sessile organisms, plants are unable to move in the process of growth, so they have evolved their own defense mechanisms in the face of fungal invasion [7]. After the failure of physical defense, plant receptors represented by PRRs (pattern recognition receptors, PRRs) activate PTI (PAMP-triggered immunity, PTI) for molecular defense by sensing the derived molecules of pathogenic fungi [8]. In their long evolution, some fungi also suppressed and escaped PTI in the way of the evolutionary hijacking of plant-sensitive factors [9–12]. In response to this change, plants evolved effector-triggered (ETI) responses to disease resistance led by R genes [13]. Most of the known resistance genes (*R* genes) belong to the NLR (nucleotide-binding domains and leucine-rich repeats) class [14]. Once a pathogenic effector is detected, NLRs will undergo a conformational transition from a concentrated ADP-binding state to an open ATP-binding state, while exposing the N-terminal domain, which is used to initiate downstream signaling [15,16]. In NLRs, the N-terminal TIR (Toll/interleukin-1-receptor-like, TIR) or CC (coiled-coil, CC) domain is preceded by an evolution-conserved domain (NB-ARC) [17], followed by a highly variable LRR domain. Nucleotide binding leucine-rich repeat (NB-LRR) in plant immune systema is an effective way for plants to resist pathogenic attack by recognizing the immune response triggered by pathogen effectors [18]. Arabidopsis *SNC1* (the suppressor of npr1-1) is a TIR-type NB-LRR (Toll/interleukin-1 receptor-NB-LRR) resistance gene. Its expression level and function are directly related to the balance of plant immune ability and growth and development. Therefore, plants need to closely control the expression of NB-LRR in vivo [19].

In the study on Arabidopsis, the copine gene *AtBON1* was found to negatively regulate the resistance of Arabidopsis to pathogens by regulating the expression of the NLR gene *SCN1* [20]. In studies on the resistance of Arabidopsis, rice, wheat, etc., the *copine* gene was silenced or downregulated, resulting in increased resistance to trophic or semi-trophic pathogens in the phenotype of plants, which proved that *copine* is a potential gene for improving plant disease resistance [21–23].

The *copine* gene can encode a class of Ca^{2+}-dependent phospholipid membrane-binding proteins, which are widely distributed in eukaryotes [24]. Copine protein was first discovered in the separation of paramecium membrane transport proteins. In the presence of Ca^{2+}, this protein can bind to the phospholipid membrane and appears as a uniform 55 kDa band during isolation. Subsequent studies have revealed that the protein has two duplicated C2 domains and one vWA domain. Some scholars observed an Arabidopsis mutant with trait changes under low temperature conditions in their research and named the mutant gene *BON1* according to the word "Bonzai" after the study [25]. Sequence studies proved that *BON1* is a gene encoding copine protein. At present, the *copine* gene has been widely studied in mammals, and some functions of this protein have been found. In mammals, eight members of the copine family have been identified, which are widely involved in physiological processes such as signal transduction, membrane transport, and cell migration [26]. In studies of mouse neurons, *copine-6* can bind to SNRAE protein in a Ca^{2+}-dependent manner and selectively inhibit spontaneous neurotransmission [27]. In Dictyostelium, *CPNA* is involved in the differentiation of stem cells and the germination of terminal buds and regulates the phototaxis and thermotaxis of its hyphae [28]. In the study of NSCLC, inhibition of *CPNE1* can inhibit the proliferation and motility of NSCLC in normal cells [29].

Few studies have been carried out on copine proteins in plants. Compared with animals, there are usually no more than three copine family members in plants, and some plants even have only one [30]. In Arabidopsis, some functions of the *copine* gene have been revealed. Two independent groups revealed the role of the copine gene *AtBON1* from two different perspectives. The former found that Arabidopsis mutant *bon1* exhibited inhibited growth at 22 °C, at low temperature, which could be relieved at 28 °C [25]. A second group of researchers found that a *con1-1* Arabidopsis mutant showed a dependence

on relative humidity, with plants growing at low relative humidity showing a growth malformation and spontaneous cell death when the sun was extended. Such mutants also produce more rapid and intense hypersensitive reactions to nutritive pathogens [31]. These studies demonstrated that *BON1* was regulated by temperature and enhanced plant hypersensitivity (HR) responses. Subsequent studies proved that this response was related to the negative regulation of *SNC1* by *BON1* [32]. Yang also found that the three *copine* genes in Arabidopsis had a certain degree of functional overlap, and *AtBON1/2/3* were necessary for the normal growth of Arabidopsis, and all of them could negatively regulate the resistance response of Arabidopsis. The growth defect phenotype in bon1-1 mutants can be compensated to some extent by overexpression of *AtBON3* [33]. With an in-depth study, Chen et al. revealed more functions of the *copine* gene in *BON* regulating plant osmotic stress [34]. In the RNA-seq analysis of *bon123t* mutant and control (WT) Arabidopsis after mannitol and ABA (Abscisic Acid, ABA) stress, it was found that 78 genes were upregulated in WT after ABA and mannitol stress, but there was no difference in *bon123t* expression, indicating that the *copine* gene plays a key role in osmotic stress. It also plays an important role in controlling ABA induction. In Arabidopsis, *bon123t* exhibits a growth-lethal phenotype, and defects in ABA accumulation can be relieved by knockdown of *PAD4*, an NLR signaling regulator, demonstrating that *BONs* exhibit a function dependent on NLR signaling [34]. In addition to Arabidopsis, the function of *copine* genes in some plants has also been revealed, especially in mediating plant disease resistance. Xin et al. showed that the rice copine genes *OsBON1* and *OsBON3* are both negative regulators of disease resistance regulation in rice. The RNAi-silenced OsBON1-RNAi showed enhanced resistance to *Pyricularia grisea* and *Xanthomonas oryzae* pv. *oryzae* (Xoo). However, after the enhanced expression of *OsBON3* in rice, the growth of transformed plants increased and showed decreased resistance to Xoo disease [22]. We report here that *copine* genes are differentially expressed between tissues, with them being upregulated in response to northern corn leaf blight as well as ABA, SA (Salicylic acid, SA), JA (jasmonic acid, JA), and low temperature, and downregulated in response to high temperature stress. Our analysis showed that ZmBON3 was involved in the regulation of functional and physiological traits such as disease resistance, plant height, grain length, and 100-grain weight. In order to better elucidate and utilize the role and function of *ZmBON3*, the objectives of this study were to (i) obtain *ZmBON3* overexpression and knockout-positive plants through a series of transgenic technologies; (ii) to investigate the agronomic traits of transgenic positive plants and the changes of related physiological and biochemical indexes; (iii) to analyze and study the changes in the transcription level of key genes related to disease resistance after inoculation of Northern Corn Leaf Blight.

2. Results

2.1. Detection of Transgenic Plants by Fluorescence Quantitative PCR

The expression of the *ZmBON3* gene in each part of the plant was detected using the cDNA of T2 generation recombinant plants with positive molecular detection as the template (Figure 1). The expression of OE lines (overexpressing plant lines) was significantly increased compared with the control, while the CR lines (expression of the ZmBON3-gene-edited plants) were decreased by 75–80% compared with the pair.

2.2. Effect of ZmBON3 on Disease Resistance of Maize Plants

The leaves of the control, OE lines, and CR lines were inoculated with Northern Corn Leaf Blight, and then left for 15 days under natural conditions for full onset. It could be found that there were clear and obvious spindle-shaped long spots on the leaves of the control plants and the OE lines (Figure 2a). A single leaf with relatively obvious lesions was selected from infected plants at maturity stage for statistical analysis. The number and total area of all diseased spots on the leaves were measured, and the average diseased spot area was obtained according to the total area/number of diseased spots. The average lesion area of the control plants was 1.0 cm^2, that of OE was 2.2 cm^2, and that of CR was

0.45 cm^2. Compared with the control plants, the area of dead spots in the OE lines was larger. However, the CR lines only had water-drop-shaped spots on the leaves, and the area of the spots decreased significantly. The CR lines were significantly enhanced in disease resistance, while the OE lines were significantly decreased in the resistance to pathogens (Figure 2b).

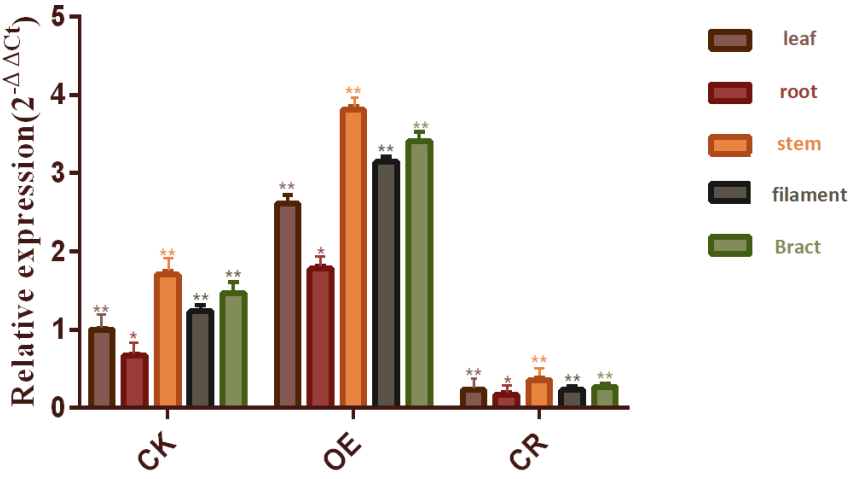

Figure 1. Relative expression of the *ZmBON3* gene in T2 generation recombination plants. Leaves, roots, and stems were taken from the three-leaf stage plants, while filaments and bracts were taken from the mature stage plants. The expression data of the same parts of CK, OE, and CR plants were selected for statistical analysis. CK: control plants; OE: transgenic plants overexpressing the *ZmBON3* gene; CR: *ZmBON3*-gene-edited plants. Each set of data was taken from the mean value of relative expression and statistically analyzed. Similar results were obtained in three separate experiments. The relative expression levels with significant differences were marked with * (* $p \leq 0.05$, ** $p \leq 0.01$).

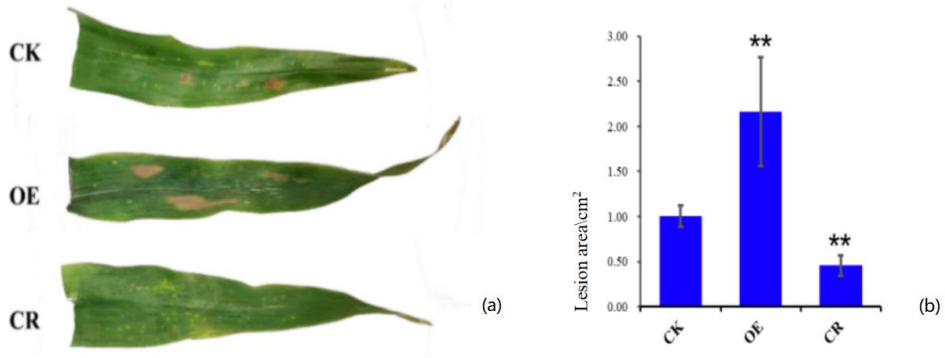

Figure 2. Incidence of large spots in transgenic plants inoculated: (**a**) incidence of leaf; (**b**) leaf spot area statistics. ** means significant difference compared with the control ($p \leq 0.01$). CK: control plants; OE: transgenic plants overexpressing the *ZmBON3* gene; CR: *ZmBON3*-gene-edited plants.

2.3. Changes in Physiological and Biochemical Parameters Related to Defense in Transgenic Plants

The results showed that H_2O_2 began to rise after induction in the leaves of each plant, and the rate of H_2O_2 rise in the CR lines was higher than that in the OE lines. The content of H_2O_2 in the CR lines had increased to 103.62 $mol^{-1}FW^{-1}$ one day after inoculation. It was 1.66 times that of the control and 1.78 times that of the OE lines, with it reaching the peak value. After that, the CR lines also maintained a high level of H_2O_2 content (Figure 3a). Compared with the control plants, the increase in the OE lines was relatively slow.

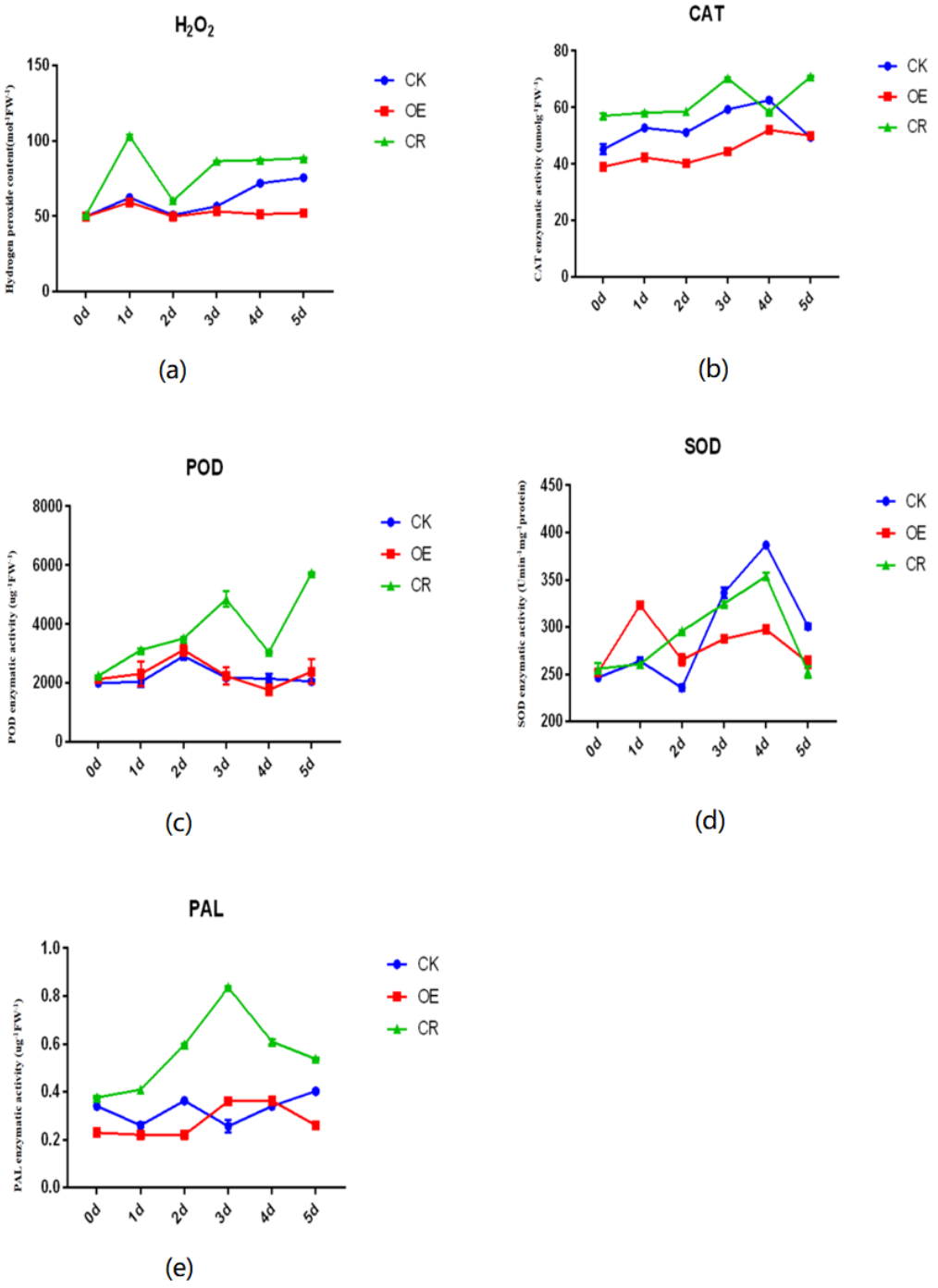

Figure 3. (**a**) Change in H_2O_2 content in transgenic plants; (**b**) changes in CAT enzyme activity in transgenic plants; (**c**) changes in POD enzyme activity in transgenic plants; (**d**) changes in SOD activity in transgenic plants; (**e**) changes in PAL enzyme activity in transgenic plants. CK: control plants; OE: transgenic plants overexpressing the *ZmBON3* gene; CR: *ZmBON3*-gene-edited plants. All of the materials were taken from trileaf-stage plants. Each set of data showed an average of the relative expression levels, and similar results were obtained in three separate experiments.

The results showed that the CAT activity of CR lines was higher than that of the control plants and the OE lines at 0 d and it reached the highest expression level at 3 d after inoculation. The CAT enzyme activity of the control plants and the OE lines increased at a slower rate and reached their peak on the 4th day (Figure 3b). The experimental results

showed that the CR lines were more sensitive to H_2O_2 and more rapid in their resistance to pathogens.

The CR lines showed significant POD (peroxidase, POD) activity at 5 days after inoculation, and this activity peaked at 5 days after inoculation. The activity of POD enzyme in OE lines and control plants was relatively stable, and the activity reached a small peak on the second day, and then returned to the original level (Figure 3c).

The SOD (superoxide dismutase, SOD) activity in the CR lines showed a trend of steady increase on the 4th day after inoculation and reached the highest level on the 4th day (Figure 3d).

The changes in PAL (L-phenylalanin ammo-nialyase, PAL) activity after inoculation were as follows: the PAL activity in the CR lines was significantly higher than that in the OE lines and the control plants, and the PAL activity began to increase substantially after the first day of inoculation and reached its peak on the third day (Figure 3e). At the peak, the PAL activity of the CR lines was 2.27 times higher than that of the control plants, indicating that the disease resistance of the CR lines was significantly improved.

2.4. Changes in Transcription Levels of Key Genes Related to Disease Resistance Pathways

More than one signal transduction pathway is usually involved in plant disease resistance response, and a variety of defense pathways also antagonize and cooperate with each other, and jointly participate in the response of plant defense response. To investigate the role of the *ZmBON3* gene in the regulation of disease resistance signaling, transcription levels of key genes in defense signaling pathways were measured in tested plants at 72 h after inoculation with Northern Corn Leaf Blight. In this experiment, the expression of *ZmSGT1* and *ZmPAD4* was significantly upregulated under the induction of Northern Corn Leaf Blight, while the CR lines were more responsive to *R* gene, and the expression of the *ZmBON3* gene was always higher than that of the control in the process of disease resistance. The expression of *ZmPAD4* and *ZmSGT1* in the CR lines peaked at 12 days after inoculation, and the values were 2.84 times and 2.25 times those of the control's expression and 5.41 times and 2.4 times those of the OE lines, respectively (Figure 4a).

The results showed that the three key genes of the SA pathway were strongly induced after northern corn leaf blight inoculation, and the intensity of expression was significantly higher in the CR lines than in the control plants. The peak expression times of *ZmPR1*, *ZmPR5*, and *ZmPAL* were different. The peak expression times of *ZMPR1*, *ZMPR5* and *ZMPAL* were 72 h, 48 h, and 24 h, respectively. The expression levels of *ZMPR1*, *ZMPR5*, and *ZMPAL* were upregulated 3.23–5.13 times compared with the control, and 4.49–6.32 times compared with the OE lines (Figure 4b).

In this experiment, in the CR lines, *ZmAOS* rapidly reached its peak at the 4 h day after inoculation, and the upregulated expression was 2.51 times of that o the control plants and 4.05 times of that in the OE lines. After that, the expression of *ZmAOS* continuously decreased to a state similar to that before stress. After inoculation of northern corn leaf blight in CR lines, the expression of *ZMPPO* remained upregulated, and the expression level was 1.38–2.81 times higher than that in the control plants (Figure 4c).

In this study, the original expression of *ZmNCED3* in the CR lines was only 0.19 times of that in control plants, and then the expression of *ZMNCED3* was also significantly inhibited after inoculation, while the effect in the OE lines was lower than that in the CR lines. After inoculation, the expression of *ZmSnRK2.6* was slightly upregulated in the control plants, but there was no significant change in the expression of *ZmSnRK2.6* in the CR lines (Figure 4d).

The above results indicated that the expression of genes in different pathways was changed in inoculated Northern Corn Leaf Blight. The expression pattern of *ZmBON3* gene was different between the CR lines and the control plants. The SA pathway, JA pathway, and *R* gene were significantly upregulated compared with the control plants, while the expression of ABA pathway genes was inhibited. In the OE lines, the gene expression pattern was similar to the control plants, but the expression degree was usually lower.

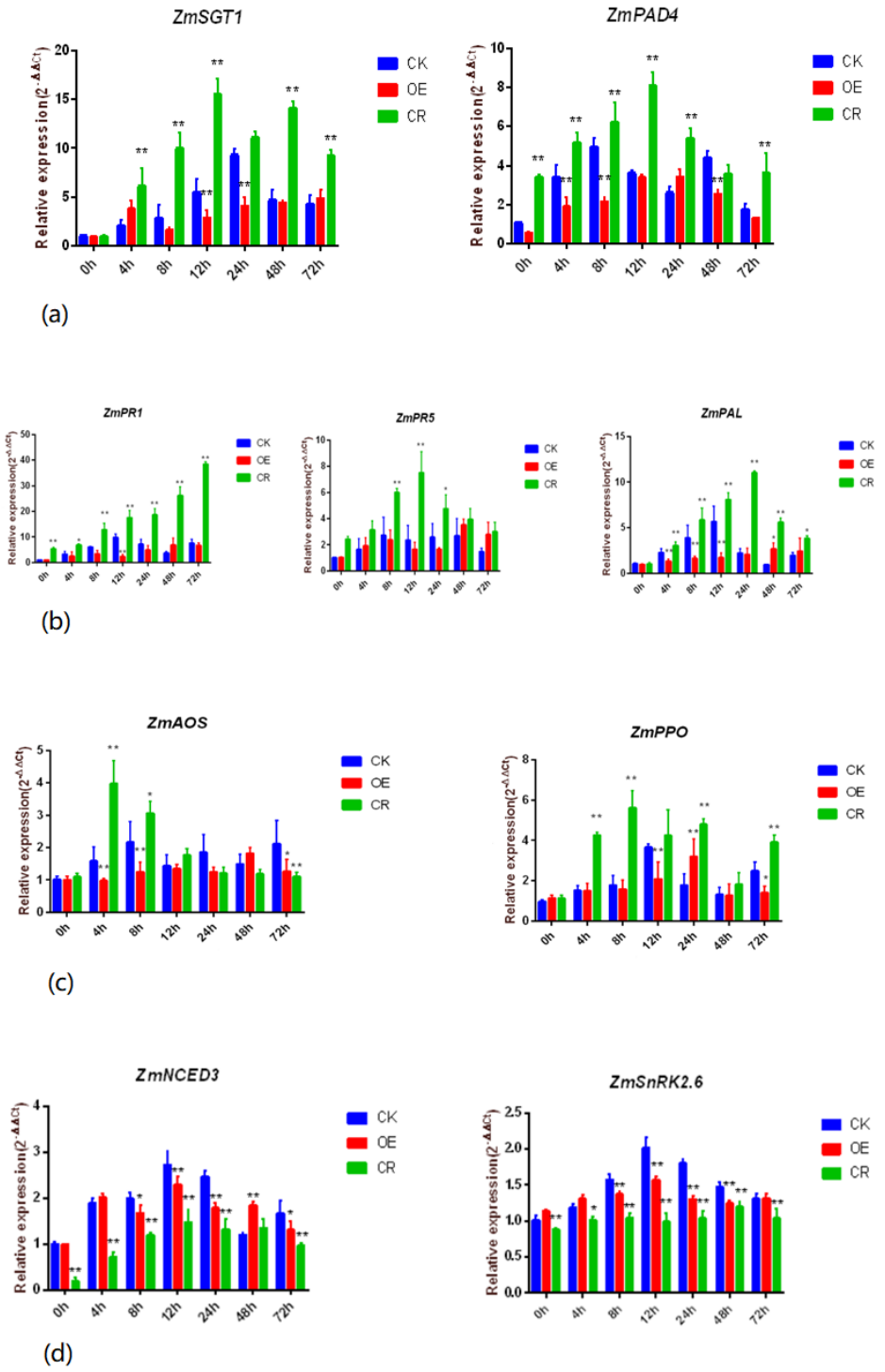

Figure 4. (**a**) *R*-gene-related gene expression changes, * means significant difference compared with the control ($p \leq 0.05$) ** means extremely significant difference compared with the contro ($p \leq 0.01$); (**b**) changes in the expression of salicylic-acid-related genes. (**c**) Jasmonate-related gene expression changes. (**d**) Abscisic-acid-related gene expression. CK: control plants; OE: transgenic plants overexpressing the *ZmBON3* gene; CR: *ZmBON3*-gene-edited plants. All of the materials are from three-leaf plants. Each set of data was taken from the mean value of relative expression and statistically analyzed. Similar results were obtained in three separate experiments.

2.5. Effect of ZmBON3 on the Spontaneous Immunity of Maize

The results of fluorescence quantification showed that the relative expression of the *PR* gene in overexpressed plants did not show much difference compared with the control. The expression of *PR1* in the CR lines was 5.42 times higher than that in the control plants and 5.84 times higher than that in the overexpressed plants. The expression of *PR5* increased 2.48 times compared with the control and 2.34 times compared with the OE lines. The expression of *ZmCAT* in the CR lines was upregulated to 4.27 times that of the control plants, while the expression of *ZmCAT* in the OE lines was slightly lower than that in the control plants and downregulated to 0.92 times that of the control plants (Figure 5a). The ROS (reactive oxygen species, ROS) accumulation level in the CR and OE lines was further detected by NBT staining. The results showed that in the absence of pathogen inoculation, no blue staining spots appeared in the control plants and the OE lines, while obvious blue spots appeared in the CR lines, which proved that ROS accumulation had begun in the CR lines (Figure 5b).

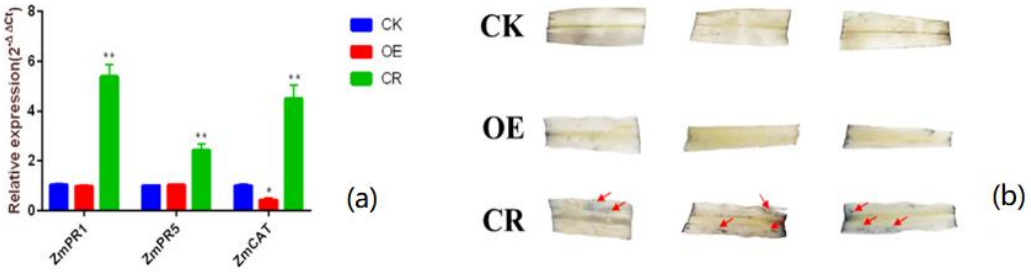

Figure 5. *ZmBON3* mediates the production of maize autoimmunity. (**a**) Expression of disease-course-related protein genes and ROS-accumulation-related genes; (**b**) NBT staining of maize leaves. The relative expression levels with significant differences were marked with * (* $p \leq 0.05$, ** $p \leq 0.01$); part of the red arrow points to the pigmented spots. CK: control plants; OE: transgenic plants overexpressing the *ZmBON3* gene; CR: *ZmBON3*-gene-edited plants.

2.6. Investigation on Agronomic Characters of Transgenic Plants

In this study, 49 strains of the T2-generation-overexpressing *ZmBON3* gene (OE lines) and 35 strains of T2 generation *ZmBON3* gene editing (CR lines) were obtained by an agrobacterium-mediated method. Significant dwarfing of the CR lines was observed at the seedling stage, and this effect persisted into future growth (Figure 6a). In the statistical analysis of agronomic traits, the plant height and 100 seed weight of the CR lines decreased significantly, while the plant height of the OE lines was less affected than that of the CR lines at the seedling stage, and the plant height was basically the same as that of the control at the statistical stage (Table 1). In addition, the OE lines showed a significant increase in 100 grain weight and grain length compared with the control (Figure 6b).

Figure 6. Agronomic traits of transgenic plants. (**a**) comparison of maize traits at the seedling stage and (**b**) Comparison of the ears and seeds of the corn. CK: control plants; OE: transgenic plants overexpressing the *ZmBON3* gene; CR: *ZmBON3*-gene-edited plants.

Table 1. Character survey of T_2 transgenic maize.

Name	Seedling Emergence	Hundred Grain Weight/g	Plant Height/cm	Ear Length/cm	Shaft Rough/cm	Grain Length/mm	Ear Rows	Grain Color
CK	88%	25.01 ± 0.71	152.66 ± 0.72	15.54 ± 0.49	3.38 ± 0.11	7.24 ± 0.38	14	orange
OE	63%	26.29 ± 0.26 **	152.39 ± 0.77	15.25 ± 0.22	3.59 ± 0.13	8.55 ± 0.34 **	14	orange
CR	54%	23.57 ± 0.49 **	146.82 ± 1.73 **	14.93 ± 0.33	3.56 ± 0.08	6.29 ± 0.17 *	14	orange

Note: * indicates a significant difference of 0.05 compared with the control; ** indicates a significant difference of 0.01 compared with the control. CK: control plants; OE: transgenic plants overexpressing the *ZmBON3* gene; CR: *ZmBON3*-gene-edited plants.

3. Discussion

copine proteins are widely found in eukaryotes. In mammals, copine proteins have eight family members, which are responsible for many important physiological functions, including membrane transport, ion transport, signal response, and so on [26]. At present, the research on the *copine* gene in plants is not perfect, and the function of the *copine* gene in some plants has been partially verified. Yang's study showed that the functions of *AtBON1*, *AtBON2*, and *AtBON3* genes of the copine family in Arabidopsis overlap, and overexpression of *AtBON3* in mutant *bon1-1* can restore the impaired phenotype in some mutants [33]. The *copine* gene is considered as a negative regulator of plant disease resistance in many plants. For example, in rice, rice OsBON1-Rnai lines silencing the *OsBON1* gene showed higher resistance to fungus blight [22]. In this experiment, inoculation of *M. corneum* could also induce the expression of *ZmBON1* and *ZmBON3*, and the expression peak was reached at 12 h after inoculation. It can be speculated that maize *ZmBON1* and *ZmBON3* may have similar functions to the *copine* gene in rice and also participate in the resistance response of maize to fungus. However, this inference is not clear, and more experiments are needed to confirm it.

The immune response of plants to pathogens is a complex physiological process involving PTI, ETI, and various signal transduction. The activation of the PR protein is usually regarded as a sign of autoimmunity activated by plant immunity. Plants recognize the components of pathogen invasion through the PR protein and bind and activate PTI to enhance plants' resistance to pathogens [35]. In this study, we found that inoculation of northern corn leaf blight could affect the expression of the *ZmBON3* gene. The transformed plants were inoculated with northern corn leaf blight to explore the role of *ZmBON3* in disease resistance. The inoculation experiments showed that the CR lines showed stronger resistance to Northern Corn Leaf Blight, and the leaf spot area on the leaf surface significantly decreased, while the OE lines showed a trend of decreasing resistance to Northern Corn Leaf Blight, indicating that *ZmBON3* may play a negative role in plant disease resistance.

H_2O_2 is a class of chemical molecules widely involved in disease resistance in plants. Plants inhibit the growth of pathogenic bacteria by increasing the content of intracellular H_2O_2, and then induce programmed cell death, so as to physically isolate the invasion of pathogenic bacteria and enhance plant resistance. This is particularly important in the resistance of trophic and semi-trophic pathogens. Catalase can efficiently remove H_2O_2 in organisms, and CAT will usually increase when H_2O_2 increases in plants. The changes of CAT enzyme activity can reflect the sensitivity of plants to H_2O_2. Peroxidase is a kind of ROS scavenging enzyme. Many studies have shown that POD (peroxidase, POD) activity in plants is related to disease resistance. Under normal circumstances, the stronger the resistance in plants, the more active the POD enzyme activity. ROS (reactive oxygen species, ROS) is a common biomolecule in plants' response to environmental stress, but a large amount of ROS accumulation often causes cell damage and cell apoptosis. SOD (superoxide dismutase, SOD) is a part of the plant system that removes ROS and protects plant growth. Therefore, the SOD activity level can be detected to judge the resistance level of plants to adversity. PAL (phenylalanine ammonia-lyase, PAL) is a key enzyme of the salicylic acid pathway and an important component of plant immune response. Jasmonic acid, as an important defense signal, is widely used in plants to resist insect feeding, the invasion

of pathogenic microorganisms, drought, and ultraviolet irradiation. Sodium alpha olefin sulfonate (SAOS) is a key gene in the pathway of jasmonic acid synthesis, and its expression is closely related to the synthesis of jasmonic acid [36]. PPO can catalyze the generation of quinones in plants to inhibit pectin decomposing enzymes and prevent the growth and invasion of pathogenic bacteria [37]. In the detection of physiological and biochemical indicators related to defense, we found that in most of the changes of defense-related indicators, the CR lines usually showed a more active defense response; in contrast, the OE lines showed a similar defense response to the control plants. After tracking the expression of some defense-related factors in the resistant plants, it was found that the content of H_2O_2, the activity of the CAT enzyme, and the activity of the PAL enzyme in the CR lines were significantly higher than those of the control and OE lines, and the response speed was faster. The high level of H_2O_2 expression may be the reason for the increased resistance in the CR lines. The changes of POD enzyme activity indicated that the CR lines may be involved in the resistance to pathogens by regulating the activity of the POD enzyme. In the activity of the CAT enzyme and the SOD enzyme, the expression in the OE lines was even lower than that in control plants. These results suggest that the *ZmBON3* gene plays a reverse regulatory role in disease resistance, which may inhibit the expression of related pathways in plant disease resistance, thereby reducing plants' resistance to pathogens.

ZmSGT1 is considered to be an important part of *ZmPAD4* related to plant R resistance, and its expression efficiency is highly correlated with plant disease resistance [38,39]. *PR1* and *PR5* are key genes in the salicylic acid pathway, involved in salicylic acid signal transduction [40] and the generation of systemic acquired resistance [41]. NCED3 is a rate-limiting enzyme in plant ABA synthesis, and its gene expression is closely related to ABA synthesis [42]. The SnRK2.6 protein is a core component of ABA signal transduction and is involved in ABA regulation of stomatal opening and closing [43]. ROS accumulation is a positive signal for plants to respond to and resist pathogen invasion. When plant autoimmunity is activated, it is usually accompanied by a higher level of ROS accumulation [44]. *PR1* and *PR5* are genes closely related to systematic programmed resistance in maize, and they play an important role in the accumulation of H_2O_2. *ZmCAT* is a component of the ROS scavenging system, and its expression will increase with the increase in plant ROS level. Through further tracking the resistance of some signal pathways in regulating gene transcription levels, the results showed two *R* genes related to the gene expression of relative controls are on the increase in the plant in the edit expression, the SA (salicylic acid, SA) and JA (jasmonic acid, JA) pathways of key gene expression under the condition of the inoculation relatively controlled plant has an obvious increase, and the expression of key genes in the ABA pathway are restrained. Mo Fengilian et al. found that after Ustilago smut infection, the contents of endogenous hormones, ABA, SA, and JA in sugarcane seedling leaves increased, and the content of ABA was low in resistant varieties, while the content of SA increased [45]. These results indicated that *ZmBON3* may affect the development of disease resistance by inhibiting *R* gene, SA, and JA pathways. In rice studies, it was found that silencing *OsBON1* led to spontaneous immune responses in plants [46]. Therefore, quantitative PCR was used to detect the expression of disease-course-related genes *PR1* and *PR5* and ROS-related gene *ZmCAT* in this study. The results showed that *PR1*, *PR5*, and *ZmCAT* genes were upregulated in CR lines. Furthermore, NBT staining experiments demonstrated that ROS accumulation had occurred in the CR lines before pathogen inoculation. The results showed that constitutive immune responses were activated in CR lines before exposure to pathogens. *ZmBON3* might be involved in the negative regulation of maize immunity, and *ZmBON3* was edited to activate the spontaneous immune response in maize.

Both high yield and high resistance are the goals of breeding, but in some studies, it has been found that the growth pathway and immune pathway of plants are usually antagonistic. Lines overexpressing *OsNPR1* in rice showed stronger resistance to rice fungus blight, but the root system, number, and weight of seeds, internode elongation rate, and number of tillers all decreased [47]. In this study, the CR lines significantly decreased

their plant height, grain length, and 100-grain weight, which may be related to the enhanced disease resistance of the plants.

4. Materials and Methods

4.1. Production of Transgenic Plants

The overexpression vector pCAMBIA-ZmBON3 and CRISPR vector pCXB053-ZmBON3 were transformed by the *Agrobacterium-tumefaciens*-mediated method. Firstly, the competent Agrobacterium strain was prepared, and then the constructed engineering vector was transformed into the competent Agrobacterium strain by the heat shock method. Callus of maize inbred line H99 was selected as the explant for genetic transformation. The transformed recombinant Agrobacterium was used to infect the explants, and the explants were induced to differentiate into seedlings after subsequent co-culture and resistance culture.

4.2. Culture and Inoculation of Northern Corn Leaf Blight

Transgenic maize plants were selected and cultured at 4 °C and 34 °C for 0 h/12 h/24 h /36 h before sampling. The pathogen stress treatment of plants was spraying, and the prepared spore suspension was sprayed and inoculated on the top four leaves, with an inoculum amount of 100 μL for each leaf. For the control group, the same amount of sterile water in the same position of the plant was sprayed. After inoculation, the culture conditions were set as 25 °C temperature, 16 h light length, and 85% relative humidity. After 14 days of culture, the disease incidence was observed and recorded.

4.3. The Transgenic Plants Were Subjected to Real-Time PCR

Total RNA was isolated from the roots, stems, leaves, filaments, and bracts of the control, OE lines, and CR lines by RNAiso Plus (TaKaRa, Changchun, China). Complementary DNA (cDNA) was synthesized using SuperScriptTM III reverse transcriptase (Invitrogen) and qRT-PCR was performed for each cDNA template. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method [48], and the analysis of each sample included three biological replicates.

4.4. Changes in Physiological and Biochemical Parameters Related to Defense in Transgenic Plants

For the determination of the H_2O_2 content. Two g of leaves to be tested were collected, flash-frozen in liquid nitrogen, and ground with a grinder. Two ml of trichloroacetic acid (concentration: 0.1%) was transferred to the sample, mixed by shaking, and placed on ice for 8 min. The centrifuge was pre-cooled until the temperature dropped to 4 °C and it was centrifuged for 10 min. The supernatant was transferred to another tube, then 0.25 of the supernatant volume of potassium phosphate buffer (10 mol/L) and 0.5 of the KI solution (1 mol/L) were added, shaken thoroughly, and then it was necessary to wait until use. the OD390 of the solution to be tested were tested and recorded.

The determination of the CAT enzyme activity. 0.2 g of fresh corn leaves were cut into pieces, and the cut material was fully frozen and ground into a powder by physical grinding. Phosphoric acid buffer (100 mmol) was added at a mass to volume ratio of 1:15, and the mixture was shaken and mixed. After centrifugation for 10 min, the supernatant was transferred to a new centrifuge tube and stored after slight centrifugation until use. To prepare the premix solution for CAT reaction, 50 mmol/L phosphate buffer and 0.1 mol/L H_2O_2 were mixed at a volume of 5:1, ddH_2O was used as a blank tube, the spectrophotometer was adjusted to 240 nm, and the prepared enzyme solution was transferred to the premix solution. The absorbance change was measured within 3 min in a period of 30 s.

Determination of peroxidase activity. The prepared plant enzyme solution was diluted with 3 mL phosphoric acid buffer (100 mmol/L) and transferred to a centrifuge tube. One ml H_2O_2 (30% concentration) and one ml guaiacol were rapidly added to the centrifuge and mixed thoroughly on a shaking centrifuge. The original enzyme solution was used as a zero-adjustment solution. The change in the absorbance value of the mixture was detected at A470, and the enzyme activity was calculated as the change of absorbance value per minute.

Determination of superoxide dismutase activity. The mixed solution for SOD activity detection was prepared in advance, and 0.2 mg NBT, 0.02 mg riboflavin, 0.2 mg EDTA-Na_2, and 4 mg methionine were weighed and mixed into 3 mL phosphate buffer solution (0.05 mol/L). After mixing, 1 mL of processed crude enzyme solution was added. After 20 min of reaction, the OD560 change was checked every minute.

Determination of PAL enzyme activity. The phenylalanine-ultraviolet absorption method was used. The preparation method of crude enzyme solution was the same as the method of CAT enzyme determination. 0.05 mL of the prepared enzyme solution was transferred to 0.1 mL of phenylalanine solution (0.6 mmol/L), and the phosphate buffer (0.1 mol/L) was used to fix the volume to 0.5 mL. After 60 min of the reaction, OD290 was detected.

4.5. Spontaneous Immune Detection and Fluorescence Quantitative Detection of Transgenic Plants

The accumulation of ROS in the target was detected by NBT staining. The first step was to prepare NBT reaction liquid: (1) weigh 50 mg NBT powder, (2) transfer 0.5 mL phosphate buffer liquid (concentration: 1 mol/L) and 0.5 mL sodium azide liquid (concentration: 1 mol/L) into the powder, (3) use deionized water to adjust the mixture to 50 mL. Second, based on the well-prepared liquid, the leaves of the plants were cut and quickly placed into the reaction liquid, with staining at room temperature (24 °C) for 90 min. At the end of staining, 95% industrial ethanol was used for decolorization at 80 °C until the green color on the surface of the material faded, and then pure water was used to wash off the ethanol two or three times. Finally, the results were recorded by taking photos.

4.6. Changes in Transcription Levels of Key Genes in Pathways Related to Disease Resistance

The key genes *ZmPR1*, *ZmPR5*, and *ZmPAL* in three SA pathways, *ZmAOS* and *ZmPPO* in two JA pathways, *ZmNCED3* and *ZmSnRK2.6* in two ABA response pathways, and *ZmSGT1* and *ZmPAD4* in two R genes, were selected [40–47]. The control, OE lines, and CR lines were infected for 72 h. Real-time fluorescence quantitative PCR was used to detect the expression levels of the related genes in leaf tissues at 0 h, 4 h, 8 h, 12 h, 24 h, 48 h, and 72 h.

4.7. Investigation on Agronomic Characters of Transgenic Plants

The positive recombinant plants were incubated in a greenhouse and then transplanted to the transgenic base of Jilin Agricultural University after reaching the four-leaf stage. Eight plants were randomly selected from each group during the growth period, and the agronomic traits of the plants were investigated after maturity.

4.8. Statistics and Analysis

The software SPSS 19.0 (SPSS Inc., Chicago, IL, United States) was used for data analysis and detection, and one-way ANOVA was used to confirm the variability of the results between the treatments. Non-significant (ns), $p < 0.05$ (*), and $p < 0.01$ (**). The experiment was repeated three times.

5. Conclusions

Through a series of biotechnology methods, such as the Agrobacterium-tumefacien- mediated method, 45 plants of T2 generation OE lines and 39 plants of CR lines were utilized in this study. Inoculation experiments of maize large spot areas showed that the CR lines showed significantly increased disease resistance, while the OE lines showed significantly decreased disease resistance. The detection of defense-related physiological indexes showed that in the detection of H_2O_2, CAT, POD, SOD, and PAL activities, CR lines > control plants > OE lines. The expression analysis of key genes of defense-related signaling pathways indicated that *ZmBON3* may be involved in the related defense processes mediated by the *R* gene, SA pathway, JA pathway, and ABA pathway. The agronomic traits of transgenic plants were investigated, and obvious dwarfing phenomenon was observed in the CR lines at the seedling stage. In terms of plant height, grain length, and 100 grain weight, CR lines < control plants < OE lines, but the ear length, axis diameter, number of ear rows, and the

grain color were not affected. We will also conduct a series of experiments on maize to observe the changes in phenotypic characteristics of transgenic maize and measure the relevant physiological and biochemical indicators. This study has laid a good foundation for elucidating the mechanism of maize disease resistance and is beneficial to maize breeding.

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