Running head: CCCH-Type Protein in Disease Resistance

Corresponding author
Shiping Wang
National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan 430070, China
E-mail: swang@mail.hzau.edu.cn
Phone: 86-27-8728-3009
Fax: 86-27-8728-7092

Research area: Plants Interacting with Other Organisms
A CCCH-Type Zinc Finger Nucleic Acid-Binding Protein Quantitatively Confers Resistance against Rice Bacterial Blight Disease

Hanqing Deng, Hongbo Liu, Xianghua Li, Jinghua Xiao, and Shiping Wang*

National Key Laboratory of Crop Genetic Improvement, National Center of Plant Gene Research (Wuhan), Huazhong Agricultural University, Wuhan 430070, China
This work was supported by grants from the National Natural Science Foundation of China (30930063) and National Program of Transgenic Variety Development of China.

*Corresponding author: swang@mail.hzau.edu.cn.
Abstract

Bacterial blight is a devastating disease of rice caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Zinc finger proteins harboring the motif with three conserved cysteine residues and one histidine residue (CCCH) belong to a large family. Although at least 67 CCCH-type zinc finger protein genes have been identified in the rice genome, their functions are poorly understood. Here we report that one of the rice CCCH-type zinc finger proteins, C3H12, containing five typical CX₈-CX₅-CX₃-H zinc finger motifs, is involved in the rice–*Xoo* interaction. Activation of *C3H12* partially enhanced resistance to *Xoo*, accompanied by the accumulation of jasmonic acid (JA) and induced expression of JA-signaling genes in rice. In contrast, knockout or suppression of *C3H12* resulted in partially increased susceptibility to *Xoo*, accompanied by decreased levels of JA and expression of JA-signaling genes in rice. *C3H12* colocalized with a minor disease resistance quantitative trait locus (QTL) to *Xoo* and the enhanced resistance of randomly chosen plants in the QTL mapping population correlated with increased expression level of *C3H12*. The C3H12 protein localized in the nucleus and possessed nucleic acid-binding activity *in vitro*. These results suggest that C3H12, as a nucleic acid-binding protein, positively and quantitatively regulates rice resistance to *Xoo* and its function is likely associated with the JA-dependent pathway.

**Key words:** DNA-binding, disease resistance, *Oryza sativa*, QTL, RNA-binding, *Xanthomonas oryzae*
A large number of genes are involved in plant resistance to pathogen invasion. These genes can be classified into two groups based on their role in defense signaling transduction, the receptor genes that include gene-for-gene disease resistance (R) genes and pattern recognition receptor (PRR) genes and defense-responsive genes (Kou and Wang, 2010). PRRs recognize pathogen-associated molecular patterns (PAMPs), which are relatively conserved during evolution, to initiate PAMP-triggered immunity (PTI) or basal resistance, whereas R proteins perceive effectors, which are more pathogen species- or race-specific as compared to PAMPs, to initiate effector-triggered immunity (ETI) or race-specific resistance (Jones and Dangl, 2006; Thomma et al., 2011). However, there is a continuum between PTI and ETI, because PAMPs and effectors as well as PRRs and R proteins cannot strictly be maintained (Thomma et al., 2011). The proteins encoded by defense-responsive genes function in the pathways initiated by PRR or R proteins.

Although different types of R, PRR, and defense-responsive genes have been characterized, the roles of CCCH-type zinc finger protein genes in host–pathogen interactions are poorly understood. CCCH-type zinc finger proteins belong to a superfamily divided into nine classes (C2H2, C8, C6, C3HC4, C2HC, C2HC5, C4, C4HC3, and CCCH) according to the numbers of conserved cysteine (C) and histidine (H) residues and the spacing between these conserved residues (Berg and Shi, 1996; Takatsuji, 1998). A CCCH-type zinc finger protein usually contains one or more tandem arranged zinc-binding motifs characterized by three cysteines followed by one histidine with the characteristics CX5-14-CX4-5-CX3-H (X indicating any amino acid) (Blackshear, 2002; Wang et al., 2008a). The CCCH-type zinc finger genes are widely present in eukaryotes (Anderson et al., 1993; Tabara et al., 1999; Carballo et al., 2000; Li et al., 2001; Gao et al., 2002; Kong et al., 2006; Guo et al., 2009) and there are at least 68 genes in Arabidopsis and 67 in rice (Wang et al., 2008a). This type of proteins has been reported to regulate gene at the posttranscriptional or transcriptional level (Li et al., 2001; Wang et al., 2008b).

Most of the characterized CCCH-type zinc finger proteins are associated with RNA metabolism, including RNA cleavage, RNA degradation, RNA polyadenylation, or RNA export by binding to RNA (Chen and Shyu, 1995; Bai and Tolias, 1996; Taylor et al., 1996; Lai et al., 1999; Lai et al., 2006; Hurt et al., 2009). In Arabidopsis, the CCCH-type
protein HUA1 is involved in the processing of AGAMOUS pre-mRNA as an RNA-binding protein during flower development (Li et al., 2001; Cheng et al., 2003). Another Arabidopsis CCCH-type protein, AtTZF1, shuttling between the nucleus and cytoplasmic foci, can bind both DNA and RNA in vitro and is likely involved in gibberellin acid/abscisic acid-mediated developmental and environmental responses through DNA or RNA regulation (Pomeranz et al., 2010).

Thus far, only a few CCCH-type zinc finger proteins have been reported to transcriptionally regulate gene expression. Arabidopsis PEI1 required for embryo development can bind to DNA and functions as an embryo-specific transcription factor (Li and Thomas, 1998). Rice OsLIC containing only one CCCH-type zinc finger motif can bind to both DNA and RNA and putatively controls plant architecture as a transcription activator (Wang et al., 2008b).

According to the strength of plant response, plant resistance against pathogen invasion is divided into two major categories: the qualitative (or complete or vertical) resistance conferred by R genes and quantitative (or partial or horizontal) resistance mediated by multiple genes or quantitative trait loci (QTLs). Quantitative resistance is frequently a broad-spectrum and durable resistance and is the only form of resistance for plants against some types of pathogens (Hu and Wang, 2009; Kou and Wang, 2010). Although a large number of resistance QTLs have been identified, only a limited number of QTLs have been characterized recently because of the genetic complexity of this type of resistance (Kou et al., 2010; Hayashi et al., 2010; Kou and Wang, 2010; Fu et al., 2011). A strategy of validation and functional analysis of the QTLs has been established to characterize resistance QTLs in rice; this strategy integrates the linkage map, expression profile, functional complementation analysis, and allele comparison and has provided the approach to characterize the genes underlying minor resistance QTLs (Hu et al., 2008; Kou et al., 2010; Kou and Wang, 2011).

Bacterial blight caused by Xanthomonas oryzae pv. oryzae (Xoo) is one of the most devastating rice diseases worldwide. A previous study revealed that rice cDNA EI38D7 (GenBank accession number BF108310), corresponding to C3H12 (locus identifier: LOC_Os01g68860; http://rice.plantbiology.msu.edu/index.shtml) based on the reported naming system for rice CCCH-type zinc finger family (Wang et al., 2008a), is a defense-responsive gene; its expression was induced in rice resistance against Xoo.
In addition, EI38D7 colocalized with a minor resistance QTL on chromosome 1 based on bioinformatic analysis (Xiong et al., 2002). These results suggest that C3H12 may be involved in quantitative resistance. To evaluate this inference, we monitored C3H12 expression, analyzed its role in rice–Xoo interaction, and co-mapped it with resistance QTLs. These analyses suggest that C3H12 encodes a nucleic acid-binding protein and appears to contribute to quantitative resistance with a small effect; its mediated resistance is associated with activation of a jasmonic acid (JA)-dependent pathway.

RESULTS

Modulating C3H12 Expression Influenced Rice Response to Xoo

Comparative analysis of the genomic and cDNA sequences reveals that C3H12 from rice variety Minghui 63 consists of seven exons and six introns and putatively encodes a protein consisting of 439 amino acids (GenBank accession number: JF799943; Supplemental Fig. S1). Sequence analysis showed that C3H12 belonged to CCCH-type zinc finger protein (Fig. 1). Functionally analyzed CCCH-type zinc finger proteins contain one to seven CCCH-type zinc finger motifs (Anderson et al., 1993; Taylor et al., 1996; Li et al., 2001; Tacahashi et al., 2003; Wang et al., 2008b; He et al., 2009; Hurt et al., 2009). The C3H12 contains five typical CX8-CX5-CX3-H zinc finger motifs (Fig. 1).

To ascertain whether C3H12 was involved in rice–Xoo interaction, C3H12 was overexpressed in susceptible rice variety Mudanjiang 8. Twenty-seven independent positive transformants, named D74UM, were obtained. Nineteen of the 27 T0 plants showed significantly enhanced (P < 0.05) resistance to Xoo strain PXO61 with lesion areas ranging from 3% to 52% (average 41%) compared to 65% for wild-type Mudanjiang 8 (Supplemental Table S1). To confirm that the enhanced resistance of the transgenic plants was due to overexpression of C3H12, four T1 families from D74UM3, D74UM17, D74UM18, and D74UM23 that all carried a single copy of transgene (Supplemental Fig. S2) were further analyzed individually for their resistance to PXO61 and the C3H12 expression level. The enhanced resistance was associated with overexpression of C3H12 in all four T1 families (Fig. 2). The correlation between disease area and C3H12 expression level in the C3H12-overexpressing (oe) plants...
shown in Figure 2 was −0.839, significant at \( \alpha = 0.01 \) (\( n = 34 \)). The growth rate of bacteria in \( C3H12 \)-oe plants was 5.4- to 10.3-fold lower than that in wild-type Mudanjiang 8 at 4 to 16 days after infection (Fig. 3A). The cosegregation of enhanced resistance and increased \( C3H12 \) expression suggest that \( C3H12 \) is involved in rice resistance against \( Xoo \).

To further examine the role of \( C3H12 \) in the rice–\( Xoo \) interaction, a \( C3H12 \)-knockout (KO) mutant (04Z11KK69), which had a T-DNA inserted into the third intron of \( C3H12 \), from the Rice Mutant Database was analyzed (Fig. 3B; Zhang et al., 2006). This \( C3H12 \)-KO line had the genetic background of Zhonghua 11 (Wu et al., 2003). We obtained 14 \( T_1 \) plants from the \( C3H12 \)-KO mutant, including eight homozygous \( C3H12 \)-KO plants, five heterozygous \( C3H12 \)-KO plants, and one wild-type sibling from the \( C3H12 \)-KO–segregating population, which were examined by PCR amplification using a gene-specific and T-DNA primer pair (Fig. 3B). The plants were inoculated with \( Xoo \) at booting stage. All the homozygous \( C3H12 \)-KO plants (2, 3, 5, 6, 9, 11, 13, and 14) showed significantly increased susceptibility (\( P < 0.05 \)) with an average lesion area of 62.4% ± 4.3% compared to 42.9% ± 3.1% for the wild-type Zhonghua 11, whereas the heterozygous \( C3H12 \)-KO plants (4, 7, 8, 10, and 12) and wild-type siblings (1) had no significant differences (\( P > 0.05 \)) from wild-type plants in response to \( Xoo \) infection (Fig. 3B). The expression of \( C3H12 \) in the homozygous \( C3H12 \)-KO plants was dramatically reduced, whereas \( C3H12 \) expression in heterozygous \( C3H12 \)-KO plants and wild-type siblings was not influenced or only partially influenced as compared to the wild-type Zhonghua 11 (Fig. 3B). The bacteria growth rate in \( C3H12 \)-KO plants was 2.7- to 5.4-fold higher than that in the wild-type Zhonghua 11 at 4 to 16 days after infection (Fig. 3A). All these results confirm that \( C3H12 \) acts as a positive regulator in rice response to \( Xoo \) infection.

**\( C3H12 \) Colocalized with a Minor Disease Resistance QTL**

Indica rice variety Minghui 63 carries the \( R \) gene \( Xa3/Xa26 \) for resistance to \( Xoo \) strain PXO61 and japonica variety Mudanjiang 8 is susceptible to PXO61 (Sun et al., 2004). However, \( Xa3/Xa26 \) can mediate a higher level and a more broad-spectrum resistance to \( Xoo \) in the Mudanjiang 8 background than in the Minghui 63 background (Cao et al., 2007; Zhou et al., 2009). We mapped \( C3H12 \) in an \( F_2 \) population developed
from a cross between Mudanjiang 8 and Minghui 63 that had been used to screen gene loci affecting genetic background-controlled disease resistance conferred by Xa3/Xa26 (Zhou et al., 2009). C3H12 colocalized with the curve peak of a minor resistance QTL against PXO61 in chromosome 1 (Fig. 4A). This QTL explained 6.3% of the phenotypic variation of resistance in the mapping population.

To further examine whether C3H12 was involved in quantitative resistance, we suppressed C3H12 in the resistant parent Minghui 63 of the mapping population using the RNA interference (RNAi) strategy. Six independent transformants, named 38Ri, were obtained. The C3H12 transcript levels in the positive C3H12-RNAi plants were 17.9% to 53.9% of that in wild-type plants. The five positive plants showed significantly increased ($P < 0.01$) susceptibility to Xoo strain PXO61 with lesion areas ranging from 21% to 25% versus 8% for the wild-type Minghui 63 (Fig. 4B). The increased susceptibility was associated with suppression of C3H12 (Fig. 4B). The correlation between disease area and C3H12 expression level in the C3H12-RNAi plants shown in Figure 4B was –0.975, significant at $\alpha = 0.01$ ($n = 6$). These results suggest that C3H12 may be involved in regulating quantitative resistance against Xoo.

However, the resistance allele at the QTL was from susceptible Mudanjiang 8. Comparative sequence analysis showed that the C3H12 alleles in Minghui 63 and Mudanjiang 8 had six nucleotide substitutions in introns (Supplemental Fig. S3); thus, the C3H12 alleles of the two rice varieties encode an identical protein. The promoter region of C3H12 in Mudanjiang 8 had 12 nucleotide substitutions and an 8-nucleotide insertion as compared with that in Minghui 63 (Supplemental Fig. S3). The C3H12 allele and its promoter in rice variety Zhonghua 11, which was also used as a recipient of transgene, had an identical sequence as that in Mudanjiang 8 (Supplemental Fig. S3). These results suggest that the C3H12 allele putatively contributing to the resistant locus may result from an expressional difference during the rice–Xoo interaction, as compared with its susceptible allele in the mapping population.

**Xoo Infection Influenced C3H12 Expression**

To test whether C3H12 had different expression patterns in resistant and susceptible reactions, we analyzed C3H12 expression in two pairs of rice lines after PXO61 infection. Rice variety Zhenshan 97 is susceptible to PXO61 and is an indica variety as
the moderate resistant Minghui 63 carrying \( R \) gene \( Xa3/Xa26 \) (Cao et al., 2007). Rb49 is a transgenic line, carrying a single copy of \( Xa3/Xa26 \) driven by its native promoter, with the genetic background of susceptible rice variety Mudanjiang 8; it is more resistant to \( Xoo \) than Minghui 63, the donor of \( Xa3/Xa26 \) (Sun et al., 2004; Cao et al., 2007). \( C3H12 \) showed a similar expression pattern in both resistant and susceptible rice lines after \( Xoo \) infection (Fig. 5). Its expression was rapidly suppressed at 1 to 12 h after infection and then returned to the basal level or was induced at 24 to 48 h after infection. However, the expression level of \( C3H12 \) was significantly higher \( (P < 0.05) \) in resistant lines than in susceptible lines either with or without pathogen infection. Interestingly, with the presence of \( Xa3/Xa26 \), the expression level of \( C3H12 \) in Mudanjiang 8 background (rice line Rb49) was significantly higher \( (P < 0.05) \) than that in Minghui 63 (Fig. 5).

Twenty-one randomly chosen \( F_2 \) plants generated from the cross between Mudanjiang 8 and Minghui 63 and segregated for \( R \) gene \( Xa3/Xa26 \) to \( Xoo \) were further analyzed for the relationship between resistance and \( C3H12 \) expression level. Increased \( C3H12 \) expression was correlated \( (r = -0.5, n = 21, \text{ significant at } \alpha = 0.05) \) with the enhanced resistance in these \( F_2 \) plants (Supplemental Fig. S4). The genotypes of some \( F_2 \) plants at the resistance loci were further analyzed using gene markers or simple sequence repeat markers flanking resistance QTLs in this \( F_2 \) population (Zhou et al., 2009). Several \( F_2 \) plants that had relatively consistent genetic backgrounds at major resistance loci (explained larger than 10% of the phenotypic variation of resistance in the mapping population) but had \( C3H12 \) allele from either of the parents were identified (Fig. 4C). Plant 43 that carried \( C3H12 \) allele from Mudanjiang 8 and had significantly increased \( C3H12 \) transcripts showed enhanced resistance to \( Xoo \) as compared to plant 11 that carried \( C3H12 \) allele from Minghui 63 and had significantly reduced \( C3H12 \) transcripts (Fig. 4C, Supplemental Fig. S4). There was a similar situation for plants 19 and 38 that carried \( C3H12 \) allele from Mudanjiang 8 as compared for plants 28 and 19 that carried \( C3H12 \) allele from Minghui 63. These results suggest that rice resistance is associated with a higher level of \( C3H12 \) transcripts and a higher level of \( C3H12 \) expression is contributed by the allele from Mudanjiang 8. This inference is also consistent with the analysis that the resistance allele at the QTL putatively contributed by \( C3H12 \) was from Mudanjiang 8 background in the mapping population (Fig. 4A).
**C3H12 Induced the Expression of a Set of Defense-Responsive Genes**

To dissect a possible defense pathway in which C3H12 was involved, we analyzed the expression of a set of pathogen-induced defense-responsive genes in different transgenic plants after infection of Xoo strain PXO61. PAL1 (phenylalanine ammonia lyase 1; GenBank accession number X16099), ICS1 (isochorismate synthase 1; AK120689), PAD4 (phytoalexin-deficient 4; CX118864), PR1a (acidic pathogenesis-related [PR] protein 1; AJ278436), and NH1 (Arabidopsis NPR1 homolog 1; AY9123983) were associated with the salicylic acid (SA)-dependent pathway (Qiu et al. 2007; Yuan et al. 2007; Shen et al. 2010). LOX (lipoxygenase; D14000) and AOS2 (allene oxide synthase 2; AY062258) are involved in JA synthesis (Peng et al. 1994; Mei et al. 2006). PR5 (P28493), PR10/PBZ1 (D38170), and Chil (chitinase 1; Q42993) appeared to function both in JA- and SA-dependent pathways (Qiu et al., 2007; Xiao et al., 2009; Shen et al., 2010). The TGA2.1 (AB051295), PLDβ1 (Phospholipase D β1; AJ419630), NRR (negative regulator of resistance; AY846391), and WRKY62 (DQ298182) were negative regulator in rice–Xoo interaction (Chern et al., 2005; Fitzgerald et al., 2005; Yamaguchi et al., 2009; Peng et al., 2008).

PXO61 infection induced the expression of LOX, AOS2, PR5, PR10, and Chil both in wild-type Mudanjiang 8 and Zhonghua 11 and transgenic plants (Fig. 6). However, the expression levels of the five genes were significantly higher (P < 0.05) in C3H12-oe plants than in wild-type plants both before and after infection. In contrast, the expression levels of the five genes were significantly lower (P < 0.05) in C3H12-KO plants than in wild-type plants either before or after infection. The expression of rTGA2.1 and OsPLDβ1 was slightly influenced after PXO61 infection in both wild-type and transgenic plants (Fig. 6). Nevertheless, the expression levels of the two genes were significantly lower (P < 0.05) in C3H12-oe plants and significantly higher (P < 0.05) in C3H12-KO plants than in corresponding wild-type plants both before and after infection. The expression of PAL1, ICS1, PAD4, PR1a, NH1, NRR, and WRKY62 in transgenic plants showed no obvious difference from that in wild-type plants (data not shown). These results suggest that C3H12 may be involved in a JA-dependent signaling pathway in the rice–Xoo interaction.
**C3H12 Promoted Accumulation of JA**

To gain further insight into the relationship between *C3H12* and a JA-dependent defense pathway, we quantified the concentrations of the endogenous JA in the leaves of the same plants used for analyzing the expression of defense-responsive genes after infection of *Xoo* strain PXO61 (Fig. 6). The endogenous level of JA was markedly induced by PXO61 infection in both *C3H12*-oe and wild-type plants, but the JA level was significantly higher (*P* < 0.05) in *C3H12*-oe plants than in wild-type Mudanjiang 8 both before and after *Xoo* infection. In contrast, the JA level was significantly lower (*P* < 0.01) in the *C3H12*-KO plants than in wild-type Zhonghua 11 after *Xoo* infection. Consistent with the expression patterns of defense-responsive genes functioning in the SA-dependent pathway, modulating *C3H12* expression did not influence the endogenous level of SA, although *Xoo* infection slightly induced SA accumulation in both transgenic and wild-type plants (Fig. 6). These results suggest that *C3H12*-mediated disease resistance may be associated with the JA-dependent pathway.

JA inhibits root elongation and this property has been frequently used in JA synthesis- and signaling-related mutant selection (Feys et al., 1994; Lorenzo et al., 2004). The effect of methyl JA (MeJA) on root development of *C3H12* transgenic plants also supports the inference that *C3H12* regulates the JA-dependent pathway. Seed germination assay showed that the root elongation of the *C3H12*-oe seeds became more sensitive to MeJA treatment than the wild-type seeds, whereas the root elongation of *C3H12*-KO seeds was less influenced by MeJA treatment than the wild-type seeds (Fig. 7). Taken together, these results suggest that *C3H12* appears to positively regulate the JA-dependent pathway.

**C3H12 Had Nucleic Acid-Binding Ability**

To examine the possible biochemical function of C3H12 protein, its subcellular localization was first analyzed by fusing the *C3H12* coding region with the green fluorescence protein (GFP) gene. The *C3H12-GFP* fusion gene was transiently expressed in onion epidermal cells. The green fluorescent signal of C3H12-GFP protein was localized predominantly in the nucleus of the cells, whereas the control GFP was uniformly presented throughout the cytoplasm (Supplemental Fig. S5). The *C3H12-GFP* fusion gene was further expressed in rice calli. Similar result as in onion...
epidermal cells was obtained. The C3H12-GFP protein was mainly localized in the nucleus of rice cells, whereas GFP was largely expressed in the cytoplasm of rice cells (Fig. 8A). These results suggest that C3H12 may function in the nucleus.

Many proteins harboring CCCH-type zinc finger motif bind to RNA or DNA to perform their functions (Hall, 2005; Wang et al., 2008b). To understand whether C3H12 had one of these activities, we performed RNA- and DNA-binding assays using recombinant C3H12 protein purified from *Escherichia coli* and a transactivation activity assay in *Saccharomyces cerevisiae*. Under moderate salt concentrations (0.1 and 0.25 M NaCl) that resemble the *in vivo* situation (Li et al., 2001), the maltose-binding protein (MBP)-tagged C3H12 bound to certain types of ribohomopolymers (poly rA, rU, rC, or rG) and DNA (Fig. 8B). In the buffer containing 0.1 M NaCl, MBP-tagged C3H12 bound to single-strand DNA, poly rA, poly rU, and poly rG but not double-strand DNA and poly rC. In the buffer containing 0.25 M NaCl, MBP-tagged C3H12 bound only to poly rA and poly rU. The control MBP did not bind to any type of nucleic acids under the same experimental conditions. As a negative rice protein control, rice GH3-2, which is an indole-3-acetic acid-amido synthetase and catalyzes the formation of indole-3-acetic acid-amino acid (Fu et al., 2011), did not bind to any type of nucleic acid (Fig. 8B). As a positive rice protein control, rice WRKY13, which is a transcription-like regulator and binds to the promoters of some defense-responsive genes (Qiu et al., 2007, 2009), bound only to double- and single-strand DNA but not any type of ribohomopolymer (Fig. 8B). In the transactivation activity assay, C3H12 showed no activity of transactivation in yeast cells as compared to the positive control, rice transcription factor OsbZIP23 (Supplemental Fig. S6). This result is consistent with that showing no conserved activation domain identified in the C3H12 protein based on bioinformatic analysis. These results suggest that C3H12 may function as a nucleic acid-binding protein.

**DISCUSSION**

Although CCCH-type zinc finger proteins belong to a large family, their functions in plants are poorly understood. Only a few CCCH-type proteins functioning in regulation of development, growth, or abiotic stress responses have been characterized...
in Arabidopsis (Li and Thomas, 1998; Li et al., 2001; Schmitz et al., 2005; Sun et al., 2007; Kim et al., 2008; Pomeranz et al., 2010) and rice (Kong et al., 2006; Wang et al., 2008b). Although the expression profiles of CCCH-type zinc finger protein genes in Arabidopsis and rice suggest that most members in one plant-specific subfamily of the CCCH-type gene family may be involved in abiotic or biotic stress tolerance (Wang et al., 2008a), the only evidence, so far, that CCCH-type protein is involved in plant–pathogen interaction is cotton GhZFP1 (Guo et al., 2009). The GhZFP1 positively regulates resistance to the fungal pathogen *Rhizoctonia solani* in tobacco, in addition to enhancing tobacco tolerance to salt stress. Here we provide the first evidence that CCCH-type zinc finger protein is also involved in rice–pathogen interaction. Rice C3H12 functions as a positive regulator to mediate resistance against the bacterial pathogen *Xoo*.

**C3H12-Mediated Disease Resistance Is Associated with Activation of the JA-Dependent Pathway**

JA and SA are two well-known phytohormones involved in host–pathogen interactions. In general, plant resistance to biotrophic and hemibiotrophic pathogens is frequently controlled by the SA-dependent pathway, whereas resistance to necrotrophic pathogens is frequently regulated by the JA/ethylene-dependent pathway (Bari and Jones, 2009). *Xoo* is a biotrophic pathogen. The present results suggest that C3H12-mediated bacterial resistance may be dependent on JA but not SA. This inference is supported by the following evidence. First, the enhanced resistance of C3H12-oe plants was associated with increased transcripts of JA synthesis-related genes (*LOX* and *AOS2*) and accumulation of JA but not with expression of SA synthesis-related genes (*PAL1* and *ICS1*) and SA signaling-related genes (*PAD4*, *PR1a*, and *NH1*) and accumulation of SA. Second, the hypersensitivity of C3H12-oe plants and hyposensitivity of C3H12–KO plants to MeJA treatment on root development suggest that C3H12 positively regulates a JA-dependent pathway.

Rice resistance against *Xoo* appears to be regulated by multiple SA- or JA-related pathways. The enhanced rice resistance to *Xoo* by activating WRKY13 or suppressing OsDR10 or MPK6 that negatively regulates systemic acquired resistance and positively regulates local resistance is associated with activation of the SA-dependent pathway and
suppression of the JA-dependent pathway (Qiu et al., 2007, 2008; Xiao et al., 2009; Shen et al., 2010). In addition, enhanced rice resistance to Xoo can be also achieved by activating MPK6 or suppressing WRKY45-1 or EDR1, which is associated with activation of both JA- and SA-dependent pathways (Tao et al., 2009; Shen et al., 2010, 2011). Furthermore, suppressing the auxin-dependent pathway by activating either GH3-2 or GH3-8 can enhance rice resistance against Xoo, which is accompanied with suppression of both SA- and JA-dependent pathways (Ding et al., 2008; Fu et al., 2011). Like the C3H12-oe plants, the enhanced rice resistance to Xoo by activating WRKY45-2, which is the allele of WRKY45-1 in indica rice, is associated with increased accumulation of JA but not SA (Tao et al., 2009). Interestingly, activation of WRKY45-2 did not significantly influence ($P > 0.05$) C3H12 expression, whereas suppression of C3H12 significantly repressed ($P < 0.01$) WRKY45-2 expression (Supplemental Fig. S7). These results suggest that C3H12 and WRKY45-2 may function in the same defense transduction pathway with WRKY45-2 localizing at the downstream of C3H12. Furthermore, multiple mechanisms may be involved in rice resistance against Xoo, although this inference needs to be confirmed by analyzing double or triple mutants.

### C3H12 May Function as an RNA-Binding Protein

Zinc finger is a characterized motif for nucleic acid binging (Hall, 2005). Most of the characterized CCCH-type zinc finger proteins are associated with RNA metabolism by binding to the target mRNA (Cheng et al., 2003; Delaney et al., 2006; Lai et al., 2006; Hurt et al., 2009) and only two, the Arabidopsis PEI1 and rice OsLIC, are suggested to transcriptionally regulate gene expression by binding to DNA (Li and Thomas, 1998; Wang et al., 2008b). In addition, OsLIC harbors an EELR-type activation domain for regulating gene transcription in yeast (Wang et al., 2008b). Consistent with other characterized CCCH-type proteins, C3H12 localized in the nucleus and possessed the capability of nucleic acid-binding, suggesting its potential role in RNA or DNA regulation. However, C3H12 did not display transactivation activity in yeast cells and preferentially bound to poly rA and poly rU but not double- and single-strand DNA in the buffer containing a relatively higher physiologic concentration of NaCl, suggesting that C3H12 may function as an RNA-binding protein. This assumption is also supported by the evidence that C3H12 does not harbor a known activation domain based on
bioinformatic analysis. According to the nuclear localization and nucleic acid-binding specificity of the C3H12 protein, a potential role in nuclear RNA regulation is considered.

C3H12 may regulate disease resistance by promoting the cleavage or degradation of mRNAs of some defense-responsive genes that encoded proteins function as negative regulators in rice–Xoo interaction and thus remove the suppression on defense positive regulators. TGA2.1 is a transcriptional regulator and a negative player in rice resistance against Xoo; it functions on the upstream of defense-responsive gene PR10, which positively regulate rice defense response, by suppressing PR10 expression (Fitzgerald et al., 2005). PLDβ1, which is appeared to be involved in phospholipid signaling, is also a negative regulator for defense response; the resistance of PLDβ1-knockdown plants is associated with increased expression of PR10 and chitinase genes including Chtl analyzed in the present study (Yamaguchi et al., 2009). C3H12-mediated resistance was accompanied with reduced TGA2.1 and PLDβ1 transcripts and increased PR10 and Chtl transcripts (Fig. 6). Thus, further studies may concentrate on whether C3H12 targets to the mRNA of some negative defense-responsive genes, such as TGA2.1 and PLDβ1, in rice defense response.

**C3H12 Confers Quantitative Resistance**

Map-based cloning is a traditional method to grip major resistance genes but is not efficient to isolate minor resistance QTLs because of their small effect on disease resistance. According to the analyses using the strategy of validation and functional analysis of the QTL (Hu et al., 2008), we argue that C3H12 contributes to a minor resistance QTL against Xoo. This inference can be supported by the following evidence. First, C3H12 tightly colocalized with the curve peak of the resistance QTL based on the mapping analysis using a segregation population. Second, C3H12 only conferred a partial (or quantitative) resistance after activating it, suggesting its small effect on disease resistance. Third, enhanced resistance correlated with increased expression level of C3H12 in F2 plants. Finally, suppressing C3H12 in the parent of the mapping population partially increased susceptibility to Xoo. As a positive regulator of rice resistance to Xoo, the function of C3H12 is associated with its transcriptional activation (Fig. 2). With the presence of R gene Xa3/Xa26, C3H12 showed a significantly higher
expression level in Mudanjiang 8 background than in Minghui 63 background. This may explain why the resistance QTL underlying $C3H12$ was contributed by the allele from Mudanjiang 8 in the mapping population developed from the cross between Mudanjiang 8 and Minghui 63.

Genetic background influences the function of $Xa3/Xa26$ in resistance against $Xoo$; Mudanjiang 8 background facilitates the function of $Xa3/Xa26$ more than does Minghui 63 background (Sun et al., 2004; Cao et al., 2007). The function of $Xa3/Xa26$ is associated with its expression level: the higher its expression, the more resistant the plant; $Xa3/Xa26$ has a higher expression level in Mudanjiang 8 than in Minghui 63 (Cao et al., 2007). The resistance QTL underlying $C3H12$ has been proposed to be one of the loci that facilitate $Xa3/Xa26$ function in Mudanjiang 8 background (Zhou et al., 2009). As discussed above $C3H12$ may be involved in RNA regulation and this gene is a potential candidate to study the differential regulation of $Xa3/Xa26$ expression in different genetic backgrounds.

Most of characterized plant resistance QTLs, including those in rice, have small effects on disease resistance, which makes it difficult to use minor resistance QTLs that explain <10% of the phenotypic variation for breeding programs by marker-assisted selection (Kou and Wang, 2010). The present results provide another example, in the limited list of the characterized resistance QTLs, that a single QTL with minor effect may be used in breeding programs for disease resistance after manipulating its expression.

CONCLUSION

$C3H12$, encoding a CCCH-type zinc finger protein with nucleic acid-binding activity, confers quantitative resistance against rice bacterial blight disease, which is associated with a JA-dependent defense pathway. This research may be a pioneer for further understanding the molecular functions of CCCH-type zinc finger proteins in plant–pathogen interactions.

MATERIALS AND METHODS

Gene Isolation and Structural Analysis
To isolate the \textit{C3H12} gene, the cDNA fragment of C3H12, the EI38D7 from rice variety of Minghui 63 (\textit{Oryza sativa} ssp. \textit{indica}) (Zhang et al., 2005), was used to screen the genomic bacterial artificial chromosome (BAC) library constructed with Minghui 63 tissues (Peng et al., 1998). A positive BAC clone, 16D13, was identified. A DNA fragment approximately 6 kb in length and harboring \textit{C3H12} was obtained from 16D13 by digestion with restriction enzyme \textit{HindIII} and subcloned into vector pUC19. The subclone sub38a harboring \textit{C3H12} was sequenced. The structure of \textit{C3H12} was determined by comparatively sequencing the genomic DNA and cDNA. The cDNA harboring the full length of the coding region was obtained using primers 38D75UF5 and 38D7stop (Supplemental Table S2), cloned into vector pGEM\textsuperscript{n}-T (Promega, Madison, WI, USA), and named 38F1c. The 5'-untranslated region was analyzed by 5'-rapid amplification of cDNA end (RACE) assays using the SMART\textsuperscript{TM} RACE cDNA Amplification Kit (TaKaRa Biotechnology, Dalian, China) using gene-specific primers (Supplemental Table S2) according to the manufacturer’s protocols. The EI38D7 contained the 3'-untranslated region of \textit{C3H12}.

\textbf{Plant Transformation}

The overexpressing construct of \textit{C3H12} was made by inserting a 6-kb DNA fragment (Supplemental Fig. S1) digested with \textit{KpnI} and \textit{BamHI} from subclone sub38a into vector pU1301, which contained a maize ubiquitin gene promoter (Cao et al., 2007). To construct an RNAi vector of \textit{C3H12}, a 538-nucleotide fragment amplified from Minghui 63 cDNA using primers 38D7RIF and 38D7RIR (Supplemental Table S2) was inserted into the pDS1301 vector (Yuan et al., 2007). The constructs were respectively introduced into \textit{Agrobacterium tumefaciens} strain EHA105 by electroporation. Agrobacterium-mediated transformation was performed according to a published protocol (Lin and Zhang, 2005). The \textit{C3H12}-oe construct was transferred into rice variety Mudanjiang 8 (\textit{Oryza sativa} ssp. \textit{japonica}). The \textit{C3H12}-RNAi construct was transferred into rice variety Minghui 63.

The copy number of transgene in plants was determined by DNA gel blot analysis using probe amplified from transformation marker gene hygromycin phosphotransferase (\textit{Hpt}) (Supplemental Table S2). Total DNA isolated from transgenic plants was digested with restriction enzyme \textit{BamHI} before electrophoresis.
Pathogen Inoculation

To evaluate bacterial blight disease, plants were inoculated with \textit{Xoo} strain PXO61 at the seedling or booting (panicle development) stage by the leaf-clipping method (Chen et al., 2002). Disease was scored by measuring the percent lesion area (lesion length/leaf length) at 2 weeks after inoculation. The bacterial growth rate in rice leaves was determined by counting colony-forming units (Sun et al., 2004).

Analysis of Gene Expression

RNA gel blot analysis was performed as described previously (Zhou et al., 2002). In brief, 20 $\mu$g total RNA was used for this analysis. The cDNA fragment (EI38D7) of \textit{C3H12} was used as a hybridization probe. For analyzing gene expression after \textit{Xoo} infection, the 2-cm leaf fragments next to bacterial infection sites were used for RNA isolation. The quantitative reverse transcription (qRT)-PCR analysis was conducted as described previously (Qiu et al., 2007) using gene-specific primers (Supplemental Table S3). The expression level of the rice actin gene was used to standardize the RNA sample for each qRT-PCR. The expression level relative to control was presented. For each gene, qRT-PCR assays were repeated at least twice, with each repeat having three replicates. When similar results were obtained in repeated experiments, only the result in one repetition was presented.

JA Treatment

Rice seeds used for germination assays were sterilized with 75% ethanol and 0.15% HgCl$_2$ and pre-germinated on the half-strength Murashige and Skoog (MS) medium for 2 days. The identically sprouted seeds were transplanted on 1/2 MS plates supplemented with MeJA or without supplementation of MeJA (mock control) for 7 days. The seedlings were photographed and the lengths of the primary roots were measured.

Subcellular Localization of \textit{C3H12}

To produce the \textit{C3H12-GFP} construct, the coding region of \textit{C3H12}, obtained by PCR using gene-specific primers (Supplemental Table S2) and cDNA clone 38F1c as template, was cloned into vector pU1391, which carries a $P_{\text{ub}10}:\text{GFP}$ cassette (Shen et al.,...
Transient expression of the fusion genes in white onion (*Allium cepa*) epidermal cells was performed by *Agrobacterium*-mediated transformation as described previously (Shen et al., 2010). The transformed epidermal cells were stained with 4,6'-diamidino-2-phenylindole (DAPI) and the image was observed using a confocal microscope. The *C3H12-GFP* construct was also expressed in rice callus cells by *Agrobacterium*-mediated transformation (Yuan et al. 2011). The sliced calli was stained with propidium iodide before observed under a confocal microscope.

**Co-Mapping of C3H12 and Resistance QTL**

An F2 population consisting of 146 individuals developed from a cross between susceptible Mudanjiang 8 and Minghui 63 was used for analyzing the colocalization of *C3H12* and resistance QTL. This population had been used to study the quantitative disease resistance to *Xoo* strain PXO61 and a molecular linkage map containing 136 markers spanning a total of 1631 cM was developed using this population (Zhou et al., 2009). *C3H12* was mapped on the molecular linkage map using a PCR-based derived cleaved amplification polymorphism sequence marker. The polymorphic PCR fragments were determined by electrophoresis of *XhoI*-digested PCR products amplified using primers 38D7dCAPSF and 38D7dCAPSR (Supplemental Table S2). QTL analysis was conducted using the computer program Windows QTL Cartographer Version 2.0 for composite interval mapping at a threshold of logarithm of odds (LOD) 2.0 (Wang and Zeng, 2003). The genotypes at resistance loci in some F2 plants of this population were analyzed by PCR amplification of polymorphic fragments using gene-specific primers (Supplemental Table S2) or primers for simple sequence repeat markers flanking resistance QTLs (Zhou et al., 2009).

**In Vitro Nucleic Acid-Binding Assay**

The coding region of *C3H12* was obtained by PCR using primers 38D7CF2 and 38D7CR2 (Supplemental Table S2) and cDNA clone 38F1c as template and was cloned into pMAL-c2X protein expression vector that harbors a MBP gene at the 5′-end of the multiple cloning site (New England Biolabs, Beijing, China). The *GH3-2* and *WRKY13* vectors were from previous studies (Qiu et al., 2007; Fu et al, 2011). The MBP-tagged C3H12, MBP, WRKY13, and GH3-2 proteins were purified from *Escherichia coli* using
QIAexpressionist (Qiagen, Valencia, CA, USA). In vitro nucleic acid-binding assay was performed as described previously (Yang et al., 1998). In brief, 500 ng proteins were incubated with 20 µL agarose bead-labeled poly rA, poly rU, poly rG, and poly rC and cellulose bead-labeled double-strand and single-strand calf thymus DNA (Sigma-Aldrich, St. Louis, MO, USA), respectively, in 500 µL RHPA binding buffer (10 mM Tris, pH 7.4, 2.5 mM MgCl₂, 0.5% Triton X-100, and 0.1 or 0.25 M NaCl) with 1 mg/mL heparin. After incubation at 4°C for 10 min, the beads were washed five times in RHPA buffer and boiled in SDS protein-loading buffer. The proteins were detected with the anti-MBP antibody (ProteinTech Group, Chicago, IL, USA) after separation by SDS-PAGE.

Transactivation Activity Assay

The transactivation activity of C3H12 was analyzed in yeast cells using the known rice transcription factor OsbZIP23 (Xiang et al., 2008) as control. The coding regions of C3H12 and rice OsbZIP23 were amplified using primer pairs 38D7CF1/38D7CR and OsbZIP23F/OsbZIP23R, respectively (Supplemental Table S2). PCR product was sequenced, digested using NcoI and BamHI, and ligated into the pGBKT7 vector. The recombinant vector and the pGBKT7 empty vector (control) were, respectively, transferred into Saccharomyces cerevisiae strain AH109 by yeast LiAc-mediated transformation according to the manufacturer’s protocol (BD Biosciences Clontech, Mountain View, CA, USA). Yeast transformants were screened by culture in SD-Trp-His-Ade medium.

Sequence Analysis

The CCCH-type zinc finger motif of C3H12 and other proteins was predicted by searching the Conserved Domains database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The sequence alignment was performed using program Genedoc version 3.2 (http://www.psc.edu/biomed/genedoc).

Statistical Analysis

The significant differences between control and treatment of the samples were analyzed by the pair-wise t test installed in the Microsoft Office Excel program. The
correlation analysis between disease area and $C3H12$ expression level was performed using the CORREL analysis installed in the Microsoft Office Excel program.

Supplemental Data

Supplemental Figure S1. The structures of $C3H12$ gene and rice DNA fragment for transformation.

Supplemental Figure S2. Southern blot analysis of the copy numbers of transgene $C3H12$.

Supplemental Figure S3. Sequence comparison of the $C3H12$ gene and its promoter region from rice varieties Minghui 63, Mudanjiang 8, and Zhonghua 11.

Supplemental Figure S4. Relationship of $C3H12$ expression level and the resistance level in F$_2$ plants.

Supplemental Figure S5. $C3H12$ localized in the nuclei of onion epidermal cells.

Supplemental Figure S6. $C3H12$ displayed no transactivation activity as compared to the positive control.

Supplemental Figure S7. $C3H12$ influenced the expression of defense-responsive gene $WRKY45-2$.

Supplemental Table S1. Resistance of T$_0$ $C3H12$-overexpressing plants (D74UM) to Xoo strain PXO61 at booting stage.

Supplemental Table S2. PCR primers used for construction of vectors, gene structure analysis, gene mapping, and transgene copy number analysis.

Supplemental Table S3. Primers used for quantitative PCR in gene expression analysis.

LITERATURE CITED


Berg JM, Shi Y (1996) The galvanization of biology: a growing appreciation for the
roles of zinc. Science 271: 1081–1085


for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl JA, and resistant to a bacterial pathogen. Plant Cell 6: 751–759


Fitzgerald HA, Canlas PE, Chern MS, Ronald PC (2005) Alteration of TGA factor activity in enhanced tolerance to Xanthomonas oryzae pv. Oryzae. Plant J 43: 335-347


Lin YJ, Zhang Q (2005) Optimising the tissue culture conditions for high efficiency
transformation of *indica* rice. Plant Cell Rep **23**: 540–547


Mei CS, Qi M, Sheng GY, Yang YL (2006) Inducible overexpression of a rice allene oxide synthase gene increases the endogenous jasmonic acid level, PR gene expression, and host resistance to fungal infection. Mol Plant Microbe Interact **19**: 1127–1137


Peng Y, Bartley LE, Chen X, Dardick C, Chern M, Ruan R, Canlas PE, Ronald PC (2008) OsWRKY62 is a negative regulator of basal and *Xa21*-mediated defense against *Xanthomonas oryzae* pv. *oryzae* in rice. Mol Plant **1**: 446–458


Schmitz RJ, Hong L, Michaels S, Amasino RM (2005) FRIGIDA-ESSENTIAL 1 interacts genetically with FRIGIDA and FRIGIDA-LIKE 1 to promote the winter-annual habit of Arabidopsis thaliana. Development **132**: 5471–5478

mitogen-activated protein kinase during the process of resistance against Xanthomonas oryzae. Plant J 64: 86–99


showing enhanced and repressed expression after pathogen infection in rice (*Oryza sativa* L.). Sci China Ser C 45: 449–467

Figure legends

Figure 1. Structural features of the C3H12 protein. A, The relative locations of CCCH-type zinc finger motifs in C3H12 protein. B, Amino acid sequence alignment of CCCH-type zinc finger motifs in C3H12 (C3H12-ZF), Arabidopsis HUA1 (HUA1-ZF; National Center for Biotechnology Information [NCBI, http://www.ncbi.nlm.nih.gov] protein accession number: NP_187874), rice OsDOS (OsDOS-ZF; Q9FU27), rice OsLIC (OsLIC-ZF; Q5Z807), cotton GhZFP1 (GhZFP1-ZF; AAX20386), cucumber CsSEF1 (CsSEF1-ZF; CAI30889), human HsTTP (HsTTP-ZF; P26651), and mouse MmTTP (MmTTP-ZF; P22893).

Figure 2. Enhanced resistance to Xoo strain PXO61 is associated with overexpression (oe) of C3H12 in four T1 families. Bars represent mean (3 to 5 replicates for lesion area and 3 replicates for gene expression) ± standard deviation. The "a" indicates that a significant difference between transgenic plants and wild-type (WT) Mudanjiang 8 was detected at \( P < 0.01 \).

Figure 3. Different transgenic plants showed different responses to Xoo infection. A, Growth of PXO61 in leaves of C3H12-overexpressing (oe; D74UM18, T3 generation) and C3H12-knockout (KO; 04Z11KK69, T2 generation) plants. Bacterial populations were determined from three leaves at each time point by counting colony-forming units (cfu). B, The increased susceptibility of C3H12-KO mutant to Xoo strain PXO347 was associated with insertion of T-DNA in C3H12 and marked suppression of C3H12 expression. Bars represent mean (3 to 5 replicates for lesion area and 3 replicates for gene expression) ± standard deviation. The "a" or "b" indicates that a significant difference between mutant plants and wild-type (WT) Zhonghua 11 was detected at \( P < 0.01 \) or \( P < 0.05 \), respectively. ATG and TAA are translation start codon and translation stop codon, respectively. The T-DNA was inserted at the 1499 site of C3H12. Arrows are PCR primers used for examination of the mutant.

Figure 4. Association of C3H12 with a bacterial resistance QTL. A, Colocalization of C3H12 and a minor resistance QTL against Xoo strain PXO61. B, Increased
susceptibility to Xoo strain PXO61 was associated with suppression of C3H12 in C3H12-RNAi (38Ri) plants; neg, negative transformant. Bars represent mean (5 replicates for lesion area and 3 replicates for gene expression) ± standard deviation. The "a" indicates that a significant difference between transgenic plants and wild-type (WT) Minghui 63 was detected at $P < 0.01$. C, F2 plants carrying C3H12 allele from Mudanjiang 8 were less susceptible to PXO61 than the plants carrying C3H12 allele from Minghui 63 at relatively consistent genetic background for other resistance loci.

**Figure 5.** C3H12 expression in response to pathogen infection. Plants were inoculated with Xoo strain PXO61 at booting stage; ck, before inoculation. Bars represent mean (3 replicates) ± standard deviation. The "a" or "b" indicates that, in the same rice line, a significant difference between Xoo-infected and noninfected plants was detected at $P < 0.01$ or $P < 0.05$, respectively. Two or one asterisks indicate that a significant difference between two rice lines with the same treatment was detected at $P < 0.01$ or $P < 0.05$, respectively.

**Figure 6.** Transcriptionally modulating C3H12 influenced the expression of a set of defense-responsive genes and accumulation of jasmonic acid. Transgenic and wild-type plants were inoculated with Xoo strain PXO61 at booting stage. Bars represent mean (3 replicates) ± standard deviation. Two or one asterisks indicate that a significant difference between transgenic and wild-type plants in the same time point was detected at $P < 0.01$ or $P < 0.05$, respectively. ck, before inoculation; FW, fresh weight.

**Figure 7.** C3H12-overexpressing (oe) and C3H12-knockout (KO) plants showed opposite response to MeJA treatment in primary root development. Two or one asterisks indicate that a significant difference between the C3H12-oe or C3H12-KO and wild-type plants in the same time point was detected at $P < 0.01$ or $P < 0.05$, respectively. Mock, without supplementation of MeJA.

**Figure 8.** Analyses of the biochemical function of C3H12. A, C3H12 localized in the nuclei of rice callus cells. 1, C3H12-GFP expression; 2, staining of cell with propidium iodide (PI) as control; 3, transmission image; 4, overlay of 1, 2 and 3; 5, GFP
expression; 6, staining of cell with PI as control; 7, transmission image; 8, overlay of 5, 6 and 7. Scale bars: 10 μm. B, The maltose-binding protein (MBP)-tagged C3H12 protein bound to various nucleic acids at buffer containing 0.1 or 0.25 M NaCl. This experiment was repeated three times and similar results were obtained. dsDNA, double-strand DNA (calf thymus DNA); ssDNA, single-strand DNA (calf thymus DNA); poly rA/U/C/G, ribohomopolymers.
Figure 1. Structural features of the C3H12 protein. A, The relative locations of CCCH-type zinc finger motifs in C3H12 protein. B, Amino acid sequence alignment of CCCH-type zinc finger motifs in C3H12 (C3H12-ZF), Arabidopsis HUA1 (HUA1-ZF; National Center for Biotechnology Information [NCBI, http://www.ncbi.nlm.nih.gov] protein accession number: NP_187874), rice OsDOS (OsDOS-ZF; Q9FU27), rice OsLIC (OsLIC-ZF; Q5Z807), cotton GhZFP1 (GhZFP1-ZF; AAX20386), cucumber CsSEF1 (CsSEF1-ZF; CAI30889), human HsTTP (HsTTP-ZF; P26651), and mouse MmTTP (MmTTP-ZF; P22893).
Figure 2. Enhanced resistance to Xoo strain PXO61 is associated with overexpression (oe) of C3H12 in four T1 families. Bars represent mean (3 to 5 replicates for lesion area and 3 replicates for gene expression) ± standard deviation. The "a" indicates that a significant difference between transgenic plants and wild-type (WT) Mudanjiang 8 was detected at $P < 0.01$. 
Figure 3. Different transgenic plants showed different responses to Xoo infection. A, Growth of PXO61 in leaves of C3H12-overexpressing (oe; D74UM18, T3 generation) and C3H12-knockout (KO; 04Z11KK69, T2 generation) plants. Bacterial populations were determined from three leaves at each time point by counting colony-forming units (cfu). B, The increased susceptibility of C3H12-KO mutant to Xoo strain PXO347 was associated with insertion of T-DNA in C3H12 and marked suppression of C3H12 expression. Bars represent mean (3 to 5 replicates for lesion area and 3 replicates for gene expression) ± standard deviation. The "a" or "b" indicates that a significant difference between mutant plants and wild-type (WT) Zhonghua 11 was detected at $P < 0.01$ or $P < 0.05$, respectively. ATG and TAA are translation start codon and translation stop codon, respectively. The T-DNA was inserted at the 1499 site of C3H12. Arrows are PCR primers used for examination of the mutant.
Figure 4. Association of C3H12 with a bacterial resistance QTL. A, Colocalization of C3H12 and a minor resistance QTL against Xoo strain PXO61. Increased susceptibility to Xoo strain PXO61 was associated with suppression of C3H12 in C3H12-RNAi (38Ri) plants; neg, negative transformant. Bars represent mean (5 replicates for lesion area and 3 replicates for gene expression) ± standard deviation. The "a" indicates that a significant difference between transgenic plants and wild-type (WT) Minghui 63 was detected at $P < 0.01$. B, F2 plants carrying C3H12 allele from Mudanjiang 8 were less susceptible to PXO61 than the plants carrying C3H12 allele from Minghui 63 at relatively consistent genetic background for other resistance loci.

### Genotype and phenotype of F2 plants

<table>
<thead>
<tr>
<th>Resistance locus (allele)</th>
<th>Variation (%)</th>
<th>Marker</th>
<th>F2 plants</th>
<th>Minghui 63</th>
<th>Mudanjiang 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H12 (B)</td>
<td>6.3</td>
<td>C3H12</td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>XR5a (A)</td>
<td>7.5 - 7.9</td>
<td>MRG0338</td>
<td>H</td>
<td>H</td>
<td>A</td>
</tr>
<tr>
<td>XR8b (A)</td>
<td>15 - 21</td>
<td>RM554</td>
<td>A</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>XR4 (B)</td>
<td>6.6</td>
<td>RM401</td>
<td>B</td>
<td>H</td>
<td>A</td>
</tr>
<tr>
<td>WRKY45 (B)</td>
<td>11 - 17</td>
<td>WRKY45</td>
<td>A</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>XR7 (A)</td>
<td>7.2</td>
<td>RM18</td>
<td>A</td>
<td>H</td>
<td>A</td>
</tr>
<tr>
<td>XR9 (A)</td>
<td>8.7</td>
<td>RM215</td>
<td>A</td>
<td>B</td>
<td>H</td>
</tr>
<tr>
<td>Xa3/Xa26 (A)</td>
<td>32 - 58</td>
<td>Xa3/Xa26</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Lesion area (%)</td>
<td></td>
<td></td>
<td>47 ± 8</td>
<td>14 ± 6</td>
<td>28 ± 6</td>
</tr>
</tbody>
</table>

*aAllele contributing to resistance; A, Minghui 63; B, Mudanjiang 8.

*bPhenotypic variation of resistance explained by each resistant locus in the F2 population (Zhou et al., 2009).

*cData represents mean (5 lesion areas) ± standard deviation.
Figure 5. *C3H12* expression in response to pathogen infection. Plants were inoculated with *Xoo* strain PXO61 at booting stage; ck, before inoculation. Bars represent mean (3 replicates) ± standard deviation. The "a" or "b" indicates that, in the same rice line, a significant difference between *Xoo*-infected and noninfected plants was detected at $P < 0.01$ or $P < 0.05$, respectively. Two or one asterisks indicate that a significant difference between two rice lines with the same treatment was detected at $P < 0.01$ or $P < 0.05$, respectively.
Figure 6. Transcriptionally modulating C3H12 influenced the expression of a set of defense-responsive genes and accumulation of jasmonic acid. Transgenic and wild-type plants were inoculated with Xoo strain PXO61 at booting stage. Bars represent mean (3 replicates) ± standard deviation. Two or one asterisks indicate that a significant difference between transgenic and wild-type plants in the same time point was detected at \( P < 0.01 \) or \( P < 0.05 \), respectively. ck, before inoculation; FW, fresh weight.
**Figure 7.** C3H12-overexpressing (oe) and C3H12-knockout (KO) plants showed opposite response to MeJA treatment in primary root development. Two or one asterisks indicate that a significant difference between the C3H12-oe or C3H12-KO and wild-type plants in the same time point was detected at $P < 0.01$ or $P < 0.05$, respectively. Mock, without supplementation of MeJA.
Figure 8. Analyses of the biochemical function of C3H12. A, C3H12 localized in the nuclei of rice callus cells. 1, C3H12-GFP expression; 2, staining of cell with propidium iodide (PI) as control; 3, transmission image; 4, overlay of 1, 2 and 3; 5, GFP expression; 6, staining of cell with PI as control; 7, transmission image; 8, overlay of 5, 6 and 7. Scale bars: 10 μm. B, The maltose-binding protein (MBP)-tagged C3H12 protein bound to various nucleic acids at buffer containing 0.1 or 0.25 M NaCl. This experiment was repeated three times and similar results were obtained. dsDNA, double-strand DNA (calf thymus DNA); ssDNA, single-strand DNA (calf thymus DNA); poly rA/U/C/G, ribohomopolymers.