The *YebN* Leader RNA Acts as a Mn^{2+} Sensor Required for the Interaction of *Xanthomonas oryzae* and Rice

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Riboswitches are RNA elements that sense metabolites and control gene expression. Recently, the *yybP-ykoY* riboswitches were found to sense manganese (Mn^{2+}) and regulate the expression of diverse genes. Here, we show that the leader RNA (a *yybP-ykoY* RNA) of *yebN* in *Xanthomonas oryzae* pv. *oryzae* also functions as a sensor of Mn^{2+} . This leader RNA detects Mn^{2+} levels in plants and is essential to *X. oryzae* pv. *oryzae* virulence. Our data also indicate that Mn^{2+} is not only required as a microelement for plant growth but also acts as a defense molecule to inhibit pathogen growth. This finding highlights that Mn^{2+} plays important roles in pathogen-plant interactions and that the *yebN* leader RNA can be a target candidate for anti-*X. oryzae* pv. *oryzae* drug development.

Manganese ions (Mn^{2+}) play important roles in bacterial growth and virulence (Papp-Wallace and Maguire 2006). Mn^{2+} imbalances cause cell metabolic disorders, and excessive Mn^{2+} accumulation is toxic to cells (Nelson 1999). Bacteria rely on highly specialized mechanisms to maintain a narrow range of cytoplasmic Mn^{2+} levels. Mn^{2+} is also an essential element for plant growth and development. Moreover, reports have shown that Mn^{2+} can enhance the resistance of rice to pathogens such as *Magnaporthe oryzae* and *Pyricularia oryzae* (Cacique et al. 2012; Peris-Peris et al. 2017; Silva et al. 2012), indicating that Mn^{2+} plays an important role in pathogen-plant interactions.

We previously reported that *YebN* is a Mn^{2+} exporter, is a key factor for the maintenance of bacterial Mn^{2+} homeostasis, and is required for the virulence of *Xanthomonas oryzae* pv. *oryzae* (Li et al. 2011). *YebN* expression is regulated by Mn^{2+} via the transcriptional regulator *MntR* (Li et al. 2011). Furthermore,

there is a leader RNA in the *yebN* transcript. This leader RNA was previously predicted to be a riboswitch and a member of the *yybP-ykoY* element family (Barrick et al. 2004), which has been reported to be a Mn^{2+} -response riboswitch in *Escherichia coli* and other bacteria (Dambach et al. 2015; Price et al. 2015).

Riboswitches are regulatory RNAs that bind small-molecule metabolites and cofactors; these factors exploit specific interactions with leader sequences to control gene expression (Barrick et al. 2004; Weinberg et al. 2010). The *yybP-ykoY* element may be the most common and widely distributed riboswitch candidate; it resides upstream of genes classified into four groups (Barrick et al. 2004). With the exception of *mntP* (Veyrier et al. 2011; Waters et al. 2011), *ygiT* (Stancik et al. 2002), and *yoaB* (Price et al. 2015), these genes have not been assigned any cellular function. Examinations of the *yybP-ykoY* RNAs from *Bacillus subtilis*, *E. coli*, and *Lactococcus lactis* have confirmed that they can sense and bind Mn^{2+} (Dambach et al. 2015; Price et al. 2015). However, the *E. coli* *ygiT* leader RNA was reported to act as a pH-responsive element (Nechooshtan et al. 2009). Interestingly, Mn^{2+} can also elicit a transcriptional response from *ygiT* (Dambach et al. 2015). The role of the *yebN* leader RNA in plant pathogen fitness and virulence remains unclear.

In this work, we studied the functions of the *yebN* leader RNA in *X. oryzae* pv. *oryzae*. We found that the *yebN* leader RNA controls both transcription and translation by detecting changes of the cellular Mn^{2+} levels and is required for *X. oryzae* pv. *oryzae* Mn^{2+} homeostasis and virulence. We also showed that the *yebN* leader RNA functions as a Mn^{2+} sensor in planta and plant Mn^{2+} might act as a defense molecule to inhibit *X. oryzae* pv. *oryzae* growth. Our finding implies that a metal ion can function as a response molecule for pathogen-host interactions.

RESULTS

The *yebN* leader RNA is vital for bacterial Mn^{2+} homeostasis.

We previously reported that *yebN* expression is regulated by Mn^{2+} via the transcriptional regulator *MntR* (Li et al. 2011; Waters et al. 2011); the long 5' untranslated region (a putative *yybP-ykoY* RNA) within the *yebN* messenger RNA (Supplementary Fig. S1) might also regulate *yebN* expression. Although the *yybP-ykoY* RNAs were identified as Mn^{2+} -binding elements in *B. subtilis*, *E. coli*, and *L. lactis* (Dambach et al. 2015; Price et al. 2015), their function in plant pathogens has not been studied. To determine whether the *yebN* leader RNA functions in *X. oryzae* pv. *oryzae*, we deleted a conserved region (Mn^{2+} sensor aptamer [MSA], +1 to +115, the transcriptional start site is denoted as +1) from the genome.

Chunxia Li and Shiyao Liu jointly contributed to this article.

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Because YebN is involved in Mn^{2+} efflux (Li et al. 2011), we performed Mn^{2+} sensitivity assays. MSA deletion (ΔMSA) caused a Mn^{2+} -sensitive phenotype similar to that of wild-type *X. oryzae* pv. *oryzae* (Fig. 1A). This result indicates that i) the *yebN* leader RNA has no function or ii) it inhibits *yebN* expression under low Mn^{2+} conditions but increases *yebN* expression under high Mn^{2+} conditions (i.e., *yebN* is constitutively expressed in ΔMSA ; discussed below). By structural prediction and comparison, conserved regions were identified in the

leader RNA. Loops A and C formed the putative Mn^{2+} -binding pocket (Price et al. 2015), and we first deleted one of these ($^{49}ACAUCU^{55}$; M3) (Fig. 2A) from the *X. oryzae* pv. *oryzae* genome, to further study the function of this leader RNA. This mutant (M3) is highly sensitive to Mn^{2+} , albeit more resistant than the deletion of the *yebN* coding region (CR) (Fig. 1A). Therefore, our results suggest that the *yebN* leader RNA is moderately important for Mn^{2+} resistance in *X. oryzae* pv. *oryzae*.

Fig. 1. The *yebN* leader RNA is vital for Mn^{2+} homeostasis in *Xanthomonas oryzae* pv. *oryzae*. **A**, Phenotypes of *X. oryzae* pv. *oryzae* wild type (PXO99), $\Delta yebN$, *yebN* leader RNA aptamer-deleted (ΔMSA) and point-mutation (M3) strains on PSA (peptone-sucrose-agar) plates. Each spot was inoculated with 2 μ l of a 10-fold dilution series (i.e., 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} -fold, from left to right) from cells in logarithmic growth phase (optical density at 600 nm = 0.8). **B**, The complementary constructs used in the following works. **C**, Mn^{2+} sensitivity assays of the *Escherichia coli* *mntP* (the *X. oryzae* pv. *oryzae* *yebN* homolog) mutant ($\Delta mntP$) and its complemented strains with different *X. oryzae* pv. *oryzae* *yebN* constructs, grown on LBA (Luria Bertani + 100 mg of ampicillin per liter) plates containing 0 (left) or 5 mM $MnCl_2$ (right). Pro = Lac1-6 promoter, V = empty vector, fl = full-length *yebN*, CR = coding region, fl-M3 = full-length *yebN* with deletion of $^{49}ACAUCU^{55}$, fl-M6 = full-length *yebN* with deletion of $^{98}GAGACCA^{104}$. **D**, Mn^{2+} sensitivity assays of *X. oryzae* pv. *oryzae* *yebN* mutant ($\Delta yebN$) and its complemented strains with different *X. oryzae* pv. *oryzae* *yebN* constructs grown on PSA plates containing 0 (left) or 2.5 mM $MnCl_2$ (right).

The leader RNA controls *yebN* expression.

To avoid the effects of the *yebN* promoter activity, which can be regulated by Mn^{2+} via the transcriptional regulator MntR (Li et al. 2011; Waters et al. 2011), we replaced the native promoter with a strong promoter, *Plac1-6* (Liu et al. 2004), which is not regulated by Mn^{2+} (Dambach et al. 2015), to further analyze the roles of the *X. oryzae* pv. *oryzae* *yebN* leader RNA in Mn^{2+} homeostasis. We found that wild-type *yebN* restored the full-

length deletion of *E. coli* *mntP*, whereas the mutations of the two conserved loops (M3, ⁴⁹ACAUACU⁵⁵ deletion and M6, ⁹⁸GAGACCA¹⁰⁴ deletion) (Fig. 2A) in the MSA did not (Fig. 1B). In *X. oryzae* pv. *oryzae*, the *lac*-promoted CR can restore the CR deletion (Li et al. 2011), but the M3 and M6 mutations in the leader RNA cannot (Fig. 1C).

To test whether the *yebN* leader RNA regulates its own expression, we used a transcriptional fusion in which the reporter

Fig. 2. The *yebN* leader RNA is essential for *yebN* expression. **A**, Schematic representation of the *yebN* leader RNA secondary structures from *Xanthomonas oryzae* pv. *oryzae*, predicted by the RNA structure software program and manually edited according to the *mntP* and *yoaB* leader RNA structures. The aptamer consists of stem loops A, B, C, and D. The conserved regions outlined in black were deleted in the M3 and M6 mutations. **B**, GusA activities of the transcriptional fusion constructs (shown on the top) in *X. oryzae* grown in rich media supplemented with 0 or 5 mM $MnSO_4$. P_{yebN} = *yebN* promoter, MSA = Mn^{2+} sensor aptamer, EXP = expression platform. **C**, β -Galactosidase activities of the transcriptional (P10) and translational (P15) fusion constructs in *Escherichia coli*. P_{lac1-6} = *lac1-6* promoter, SD = the Shine-Dalgarno sequence. **D**, The effects of mutations in the leader RNA (depicted in A) on *X. oryzae* pv. *oryzae* *yebN* expression. The expression levels were determined by the derivatives of the P1 *gusA* fusion.

gene (*gusA*) (containing *gusA* CR and Shine-Dalgarno sequences) was controlled by the promoter and leader RNA of *X. oryzae* pv. *oryzae yebN* (Fig. 2B). By comparing transcriptional levels under normal (peptone-glucose-agar [PGA] medium) and high Mn^{2+} (PGA + 5 mM $MnSO_4$) levels, we found that high Mn^{2+} levels led to a 13-fold increase in *gusA* expression levels in P1 (Fig. 2B). In the absence of the MSA (P3) or in the absence of the expression platform (+116 to +183) (P4), *gusA* was induced threefold (P3 and P4) by high Mn^{2+} levels (Fig. 2B); this result is similar to that observed in fusions harboring only the *yebN* promoter (P5) (Fig. 2B), indicating that the *yebN* leader RNA is important for *yebN* expression. Together, these data indicate that the leader RNA is involved in the regulation of *yebN* expression.

The leader RNA regulates *yebN* transcription and translation.

Some studies have shown that *yypP-ykoY* RNAs regulate gene expression by different methods in different bacteria. These RNAs control translation in *E. coli* but transcription in *L. lactis* (Dambach et al. 2015; Price et al. 2015). To test whether the leader RNA regulates *yebN* transcription or translation, we used transcriptional fusion (P10) and translational fusion (P15), in which the reporter gene (*lacZ*) was controlled by the *PlacI*-6 promoter and the leader RNAs of *yebN*, to determine the expression levels in *E. coli* (Fig. 2C).

A 5.6-fold increase in *LacZ* activity was observed at high Mn^{2+} levels in P10 (Fig. 2C). In the P15 fusion, high Mn^{2+} levels led to an 11-fold increase in the levels of *lacZ* translation. The deletion of the complete aptamer domain (P16) abolished *lacZ* induction by high Mn^{2+} levels, albeit the basal expression level increased (Fig. 2C). These data indicate that the *yebN* leader RNA controls *yebN* expression levels by Mn^{2+} in both transcription and translation.

The conserved structure is essential to *yebN* leader RNA functions.

To test whether the highly conserved structure of the *yebN* leader RNA is required for *yebN* expression, a series of mutations were introduced into the RNA sequence (Fig. 2A). The effects of these mutations on reporter gene (*gusA*) induction in the presence of Mn^{2+} were determined using reporter assays (Fig. 2D). A mutation (M2) that disrupted stem A by replacing the $^{22}UUU^{24}$ sequence with $^{22}AAA^{24}$ exhibited significantly reduced reporter gene (*gusA*) expression. Similarly, point mutations that disrupted stem C (M4, in which $^{56}GCCG^{59}$ is substituted for $^{56}CGGC^{59}$) and stem D (M5, in which $^{90}CCAGA^{94}$ is substituted for $^{90}GGUCU^{94}$) caused significantly reduced levels of *gusA* induction. Compensatory mutations designed to restore these helices (M2', M4' and M5') restored Mn^{2+} induction (Fig. 2D), supporting the B, C and D stems (Fig. 2A), as shown in the structural analyses of the *mntP* and *yaoB* leader RNAs (Dambach et al. 2015; Price et al. 2015). The deletion of loop C (M3), whose sequence ($^{49}ACAUACU^{55}$) is highly structurally conserved among the *yypP-ykoY* leader RNAs, abolished *gusA* induction. The deletion of another highly conserved loop ($^{98}GAGACCA^{104}$) (M6) resulted in the loss of the ability of the leader RNA to respond to high Mn^{2+} levels (Fig. 2D). These data show that the conserved structure of the *yebN* leader RNA is required for Mn^{2+} responsiveness.

YebN leader RNA is required for *X. oryzae* pv. *oryzae* virulence.

As *YebN* is essential to *X. oryzae* pv. *oryzae* virulence in rice (Li et al. 2011), we wondered if its leader RNA can also influence *X. oryzae* pv. *oryzae* pathogenesis. We compared the virulence of the wild type, the *yebN* CR deletion mutant ($\Delta yebN$), ΔMSA , and *M3* ($^{49}ACAUACU^{55}$ deletion). In line with our previous reports, $\Delta yebN$ had no infection ability (Li

et al. 2011). ΔMSA had a lesion length similar to wild-type PXO99. Compared with PXO99 and ΔMSA , *M3* showed decreased virulence (Fig. 3), suggesting that the leader RNA is required for *X. oryzae* pv. *oryzae* virulence.

YebN leader RNA functions as a sensor for Mn^{2+} in planta.

As discussed above, the leader RNA and the coding protein of *yebN* are both required for *X. oryzae* pv. *oryzae* pathogenesis; therefore, we speculated that the leader RNA can sense the Mn^{2+} levels in planta. To this end, we first analyzed the expression levels of the *yebN* leader RNA and coding RNAs of wild type (PXO99) and a Mn^{2+} -importer mutant ($\Delta mntH$) *X. oryzae* pv. *oryzae* after infection of rice leaves. The levels of both leader and coding RNAs increased after infection, but the coding RNA had a rapid increase, indicating that the leader RNA might quickly respond to the infection-induced signals. However, the *yebN* leader and coding RNAs were not significantly changed during $\Delta mntH$ infection. Because *MntH* is the major Mn^{2+} importer and because the *yebN* leader RNA specially responds to Mn^{2+} (Fig. 4A), the *yebN* leader RNA should act as a Mn^{2+} sensor in planta. Next, we constructed a strain (*Xo05*) that expresses the *yebN* leader and coding RNAs regulated by the constitutive promoter *Lac* in $\Delta yebN$. Then, we grew rice plants (IR24) in low- and high- Mn^{2+} media and infected them with *Xo05*. We found that the levels of *yebN* leader RNAs did not significantly change in either the low or high Mn^{2+} -cultured plants, but the coding RNAs were dramatically increased in high Mn^{2+} -cultured plants, which was not seen when loop 3 (Fig. 2A) was deleted (strain *Xo06*) (Fig. 4B and C). Finally, we compared the growth rates of *Xo05* and *Xo06* in low and high Mn^{2+} -cultured plants. *Xo05* multiplied faster in low Mn^{2+} -cultured plants than in high Mn^{2+} -cultured plants. Moreover, *Xo06* grew very slowly and had no detectable difference under both low and high Mn^{2+} conditions (Fig. 4D). Collectively, these data indicate that the leader RNA can function as a Mn^{2+} sensor for *X. oryzae* pv. *oryzae*-rice interactions.

DISCUSSION

Mn^{2+} is a microelement for both pathogen and host growth. *YebN* is a Mn^{2+} exporter that is also essential to the virulence of *X. oryzae* pv. *oryzae* (Li et al. 2011). However, the *yebN* mutant of *X. campestris* pv. *campestris* had the same virulence as the wild-type strain (*X. campestris* pv. *campestris* 8004) in cabbage (Supplementary Fig. S3). Although the *X. oryzae* pv. *oryzae yebN* mutant had higher Mn^{2+} sensitivity than that of the *X. campestris* pv. *campestris* counterpart (Fig. 1A), the dramatic difference of virulence between the *X. oryzae* pv. *oryzae* and *X. campestris* pv. *campestris yebN* mutants indicates that Mn^{2+} levels might be an important host factor that regulates pathogen infection. Therefore, Mn^{2+} may act as a defense factor inhibiting pathogen multiplication in rice. Because we cannot determine the Mn^{2+} content in the infection tissues, we chose a hydroponic method to control the Mn^{2+} levels in rice leaves, in order to analyze the effect of Mn^{2+} content on *X. oryzae* pv. *oryzae* infection. Because either low (<0.1 μM) or high (>15 μM) levels of Mn^{2+} can inhibit rice growth, we used 0.2 and 10 μM concentrations as the low and high standards, respectively, in the assays. The results showed that high Mn^{2+} -cultured plants had enhanced disease resistance to the complementary strain (*Xo05*) containing wild-type *yebN* driven by the *lac* promoter but had no significant effects on the strain (*Xo06*) with the leader RNA mutation (M3) (Fig. 4). Furthermore, the expression of the *yebN* CR is induced via the leader RNA when the media has high Mn^{2+} levels (Fig. 4). These results suggest that the *yebN* leader RNA might sense the plant Mn^{2+} levels and controls *yebN* expression and that high Mn^{2+} in planta can decrease the *X. oryzae* pv. *oryzae* growth rate.

Because the leader RNA regulates both transcription and translation, inhibition of the binding of Mn^{2+} to the leader RNA can limit *X. oryzae* pv. *oryzae* growth in planta, which provides a strategy for anti-*X. oryzae* pv. *oryzae* drug development.

Understanding the mechanism of plant-pathogen interactions is vital for plant disease control. Metals are known to be essential for plant growth and development; however, some transporters of metal ions have been reported to regulate plant resistance to pathogens. For example, the NRAMP (natural resistance-associated macrophage protein) transporters AtNRAMP3 and AtNRAMP4 regulate plant basal resistance to the bacterial pathogen *Erwinia chrysanthemi* (Segond et al. 2009) and OsNRAMP6 (Mn^{2+} and iron transporter) confers disease resistance to the fungal pathogen *Magnaporthe oryzae* on rice (Peris-Peris et al. 2017). However, the role of metals in plant-microbe interactions is still unclear. Whether metals only regulate the expression of pathogenicity and virulence genes in plants or can act as signals for plant defenses to control pathogen growth in planta remains unknown. Our findings indicate that the bacterial pathogen *X. oryzae* pv. *oryzae* can sense Mn^{2+} content in planta using an RNA sensor and that plant Mn^{2+} functions as a defense molecule inhibiting *X. oryzae* pv. *oryzae* growth. During the *X. oryzae* pv. *oryzae*-rice interaction, the plant first uses the Mn^{2+} microelements to inhibit bacterial growth; then, the pathogen senses the signal and quickly synthesizes the corresponding transporter YebN to export excess

Mn^{2+} to cope with the toxicity of Mn^{2+} . The identification of plant transporters required for Mn^{2+} efflux upon *X. oryzae* pv. *oryzae* infection should be addressed in future studies.

Conclusions.

Our current findings, when combined with previous results (Li et al. 2011), support a proposed mechanistic model for Mn^{2+} homeostasis in *X. oryzae* pv. *oryzae* (Fig. 5). Expression of the Mn^{2+} uptake system gene *mntH* is negatively regulated by MntR in response to high Mn^{2+} levels (Li et al. 2011). In contrast, *yebN* promoter activity is positively regulated by MntR in response to high Mn^{2+} levels (Li et al. 2011), and the *yebN* leader RNA acts as a sensor to direct *yebN* transcription and translation by detecting Mn^{2+} levels regulated by the environment (including the host) (Fig. 5). This regulatory mechanism demonstrates that bacteria can use a transcriptional regulator and an RNA regulatory element to sense host Mn^{2+} levels and fine-tune *yebN* expression, which enables pathogens to quickly and effectively adapt to the host environment.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and growth conditions.

The bacterial strains and plasmids used in this study are listed in Supplementary Table S1 and the primers used are listed in

Fig. 3. The *yebN* leader RNA is required for *Xanthomonas oryzae* pv. *oryzae* virulence. **A**, The leaves of 60-day-old rice plants (IR24, susceptible) were inoculated with *X. oryzae* pv. *oryzae* strains or mock-inoculated with water (double-distilled H₂O). Two weeks postinoculation, images of the infected rice leaves were acquired. Yellowing represents bacterial blight lesions. WT = wild type, $\Delta yebN$ = *yebN* deletion mutant, ΔMSA = Mn^{2+} sensor aptamer deletion mutant, RSM3 = ⁴⁹ACAUAUCU⁵⁵ deletion mutant. **B**, The lesion lengths of the infected rice leaves at 2 weeks postinoculation. Virulence assays were performed in triplicate, and the means $\pm$ standard deviation were calculated (an asterisk [*] indicates $P < 0.01$).

Supplementary Table S2. Unless otherwise stated, *X. oryzae* pv. *oryzae* was grown at 28°C in PSA (peptone-sucrose-agar), PGA, or M4 minimal (MX) medium (pH 7.0) as previously described (Li et al. 2011), and *E. coli* was grown at 37°C in Luria Bertani

(LB) or ME minimal medium (12.8 g of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ per liter; 3 g of KH_2PO_4 per liter; 0.5 g of NaCl per liter; 1 g of NH_4Cl per liter; 100 μM MgCl_2 ; 0.2% glucose [vol/vol]) supplemented with the indicated chemicals.

Fig. 4. The *yebN* leader RNA functions as a sensor of plant Mn^{2+} . **A**, The relative expression levels of the *yebN* leader and coding RNA during *Xanthomonas oryzae* pv. *oryzae* (Pxo99 [WT] and *mntH* mutant) infection. **B** and **C**, The *yebN* leader and coding RNA levels after *Xo05* (**B**) and *Xo06* (**C**) infection in low (0.2 μM)- and high (10 μM)- MnCl_2 cultured rice seedlings. **D**, The growth rates of *Xo05* and *Xo06* in low (0.2 μM)- and high (10 μM)- MnCl_2 cultured rice leaves. MPI = minutes postinoculation, DPI = days postinoculation. Triplicate experiments were performed, and the means $\pm$ standard deviation were calculated.

Construction of deletion mutants and virulence analysis.

Deletion strains were constructed using the two-step inactivation method as previously described (Li et al. 2011). The virulence of *X. oryzae* pv. *oryzae* was determined as previously described (Li et al. 2011). The rice seedlings were grown by a hydroponic method (Rout and Das 2002). The growth media consisted of 4.0 mM CaNO₃, 2.0 mM MgSO₄, 4.0 mM KNO₃, 0.4 mM (NH₄)₂SO₄, 0.3 μM CuSO₄, 0.8 μM ZnSO₄, 30 μM NaCl, 0.1 μM Na₂MoO₄, 1.43 mM KH₂PO₄, 10 μM H₃BO₃, and 20 μM Fe-Na-EDTA supplemented with 0.2 (low) or 10 μM (high) MnCl₂. The in-planta growth of *X. oryzae* pv. *oryzae* was determined, as previously described (Li et al. 2011), using the hydroponically grown rice inoculated by injection (optical density at 600 nm [OD₆₀₀] = 0.2).

Determination of the *yebN* transcription start site.

Total RNA of *X. oryzae* pv. *oryzae* was extracted as described previously (Li et al. 2011). The 5' end of *yebN* mRNA was

detected using the 5' RACE System for Rapid Amplification of cDNA Ends kit (Invitrogen), according to the manufacturer's specifications. The third-cycle polymerase chain reaction (PCR) products were cloned into the pGEM-Teasy vector (Promega) and were sequenced with the M13 forward primer. Twelve clones were sequenced, and 11 of them indicated that the transcription start site was the first guanosine (underlined) of the CTCGCC CCTTGGGG sequence.

β-galactosidase (*LacZ*) activity.

β-Galactosidase activity was determined as previously described (Cromie et al. 2006). Briefly, cells were grown in LB or ME media supplemented with the indicated metal ions or other molecules. The culture density was measured (OD₆₀₀). A total of 1 × 10⁶ to 1 × 10⁷ cells were harvested by centrifugation (13,400 × g 2 min, 4°C). The supernatants were discarded, and the pellets were resuspended in 1 ml of Z buffer (60 mM

Extracellular or host environment

Fig. 5. A model of the regulation of *yebN* expression in response to plant Mn²⁺ stress. When a plant is infected by *Xanthomonas oryzae* pv. *oryzae*, it rapidly secretes Mn²⁺ into the environment of pathogen growth. Mn²⁺ is then transported by *X. oryzae* pv. *oryzae* MntH from the periplasm into the cytoplasm, in which it binds to MntR and, thus, inhibits the expression of *mntR* and *mntH*, resulting in decreased Mn²⁺ uptake. Mn²⁺-MntR also binds to the *yebN* promoter and stimulates its transcriptional initiation. Excess Mn²⁺ also quickly binds to the *yebN* leader RNA and changes the RNA structure; thus, transcription can progress, possibly leading to structural alterations of the Shine-Dalgarno sequence and the translational start region and increasing *yebN* translation levels. Under low-Mn²⁺ conditions, the leader RNA is also expressed at low levels, so it can quickly bind Mn²⁺ upon *X. oryzae* pv. *oryzae* infection and the bacteria can rapidly synthesize YebN to cope with Mn²⁺ toxicity. Solid lines indicate regions of the model supported by the current results; dashed lines indicate regions where no direct evidence has been obtained. The half arrows to the left and right indicate transcription and translation, respectively. Angled arrowheads indicate two or more molecules combined into one complex.

Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, and 50 mM β-mercaptoethanol). After cell lysis by sonication, the reaction was started by adding 0.2 ml of 2-nitrophenyl-β-D-galactopyranoside (4 mg/ml), and the reaction was stopped by adding 0.5 ml of Na₂CO₃ (1 M) stock solution when the sample in the tube developed a pale yellow color. Then, the OD at 420 nm was measured. Assays were performed in triplicate; one Miller unit (U) is expressed as 1,000 × OD₄₂₀/(OD₆₀₀ of the assayed culture × the volume assayed × time).

β-glucuronidase (GUS) activity.

For GUS assays, *X. oryzae* pv. *oryzae* strains were grown in PGA or MX media supplemented with the indicated metal ions and were harvested by centrifugation at 4°C. The supernatants were discarded, and the pellets were lysed in the GUS extraction buffer (50 mM NaHPO₄ [pH 7.0], 10 mM 2-mercaptoethanol, 10 mM Na₂EDTA, 0.1% sodium lauryl sarcosine, and 0.1% Triton X-100) by sonication. After centrifugation (13,400 × g 15 min) at 4°C, the supernatant was used for the enzyme activity assay with 4-methylumbelliferyl-β-D-glucuronide as a substrate, as previously described (Jefferson et al. 1987). Assays were performed in triplicate, and 1 U of GUS activity is expressed as nanomoles of product released per minute per 10⁸ cells. Details about the bacterial strains, plasmids construction, and primers used are provided in Supplementary Text S1.

RNA isolation and real-time reverse transcription (RT)-PCR.

Total RNA was isolated from rice leaves after *X. oryzae* pv. *oryzae* infection by injection, as previously described (Kalinowska et al. 2012). cDNAs were synthesized using a first-strand cDNA synthesis kit (TaKaRa PrimeScript) according to the manufacturer's instructions. Real-time RT-PCR was used to analyze the RNA levels of the *yebN* leader and coding RNA. The *rpoD* gene was used as the reference.

LITERATURE CITED

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