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A Sfp-type phosphopantetheinyl transferase ZmsO is essential for zeamines production and the virulence of *Dickeya zeae*

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Abstract Zeamines are family of potent antibiotics and virulence determinants produced by the rice foot rot bacterial pathogen *Dickeya zeae*. So zeamines are important for the pathogenesis of *D. zeae* and development of new strategies against this devastating disease. In this study, we show that production of zeamines is positively modulated by ZmsO, which is a conserved Sfp-type phosphopantetheinyl thransferase (PPTase) associated with post-translational activation of fatty acid synthases and polyketide synthases. Deletion of *zmsO* significantly abolished zeamines production and attenuated the virulence of *D. zeae* without affect the growth rate. The *zmsO* gene is located at the upstream of *zmsA* and *zmsK* in the same gene cluster. Consistent with its role in post-translational modification, deletion of *zmsO* did not affect the transcriptional expression of *zmsA* and *zmsK*. *In trans* expression of the *pcpS* gene from *Pseudomonas aeruginosa*, which encodes a Sfp-type PPTase, in the *zmsO* deletion mutant could also fully restore the zeamines production and bacterial virulence, establishing the important role of Sfp-type PPTase in the zeamines biosynthesis and pathogenicity of *D. zeae*.

Keywords PPTase · Zeamines · Antibiotics · Sfp-type

Introduction

Rice foot rot is a serious disease caused by *Dickeya zeae* (originally known as *Erwinia chrysanthemi* pv. *zeae*), which usually results in a substantial reduction of rice production, or even death of rice plants. The incidence of rice plants is between 20 %–100 %, and caused about 12 %–100 % reduction of yield in China. *D. zeae* is able to infect both monocotyledons and dicotyledons, while most other *Dickeya* species could only infect dicotyledons (Goto 1979; Hussain et al. 2008; Sinha and Prasad 1977). Phylogenetic analysis of *Dickeya* species showed that *D. zeae* is most distinct from other species and contains more sequevars than others (Parkinson et al. 2009). This genetic diversity may explain the wide distribution of *D. zeae*, and the diseases caused by this bacterial pathogen have been documented in many different regions and countries around the world (Sabet 1954; Jafra et al. 2009; Slawiak et al. 2009; Tsror (Lahkim) et al. 2009; Laurila et al. 2008).

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Our curiosity on the host range determinants and pathogenic mechanisms of *D. zeae* has led to identification of zeamine and zeamine II, which are structurally similar polyketide molecules with potent antimicrobial activities (Wu et al. 2010; Zhou et al. 2011; Liao et al. 2015). In addition, zeamines are also important phythotoxins which inhibit rice seeds germination, and accelerate disease symptom development in potato and Chinese cabbages (Zhou et al. 2011). Disruption of zeamines production significantly decreases the pathogenicity of *D. zeae* on rice seeds, Chinese cabbage and potato (Zhou et al. 2011; Cheng et al. 2013). Two genes associated with the biosynthesis of zeamines in *D. zeae* have been characterized recently with *zmsA* encoding a polyketide synthase (Zhou et al. 2011), and *zmsK* encoding a nonribosomal peptide synthase (Cheng et al. 2013). Zeamines have also been identified from *Serratia plymuthica* RVH1, and a gene cluster containing 18 ORFs has been designated responsible for zeamines production (Masschelein et al. 2013). Among them, *zmn10* and *zmn19* from this gene cluster share about 66 % and 48 % identity to *zmsA* and *zmsK* from *D. zeae*, respectively (Masschelein et al. 2015), which may suggest a similar evolutionary origin and synthetic pathway existing in both bacterial species.

Evidence is accumulating that post-translational modification of enzymes is necessary for biosynthesis of natural products by fatty acid synthases (Orikasa et al. 2006), polyketide synthases (Murugan and Liang 2008), and nonribosomal peptide synthetases (Liu et al. 2005). This modification is carried out by transformation of 4′-phosphopantetheine moiety from coenzyme A (CoA) to apo-carrier proteins (CPs) to form holo-carrier proteins by phosphopantetheinyl transferase (PPTase) (Beld et al. 2014; Sunbul et al. 2009). According to the substrate specificity, PPTases were classified into three subtypes. ACPS-type is involved in primary metabolism for survival, and the Sfp-type with broad specificities is related to the biosynthesis of secondary metabolites (Sanchez et al. 2001; Lambalot et al. 1996). Comparative analysis showed that ACPS-type PPTases usually contain about 120 amino acids carrying motif P2 [(V/I)G(V/I)D] and motif P3 [(F/W)(S/C/T/)xKE(A/S)xxK] (Lambalot et al. 1996), whereas the Sfp-type PPTases contain about 220 amino acids with motifs P2 and P3 plus motif P1 [PxWPxGxxGS(M/L)THCxGY] (Sanchez et al. 2001). The third type of PPTases is fused with fatty acid synthases or polyketide synthases as a functional domain (Murugan and Liang 2008; Zhang et al. 2008).

In order to reveal more information about the biosynthesis mechanisms of zeamines in *D. zeae*, we identified a phosphopantetheinyl transferase gene *zmsO*, which is essential for the production of zeamines. Null mutation of *zmsO* blocks the antibiotic activity and decreases the pathogenicity of *D. zeae*. Bioinformatics and functional analysis showed that ZmsO is a Sfp-type 4′-phosphopantetheinyl transferase. To our knowledge, this is the first report of a Sfp-type PPTase associated with the production of zeamines.

Materials And methods

Bacterial strains and growth conditions

Relative strains in this paper were listed in Table 1.

LB medium which contains (per liter) 10 g tryptone, 10 g NaCl and 5 g yeast extract (pH 7.0) and minimal medium (MM) consisting of (per liter) 10.5 g K_2HPO_4, 4.5 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 2 g mannitol, 2 g glycerol, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.01 g $CaCl_2$, 0.005 g $FeSO_4$ and 0.002 g $MnCl_2$ (pH 7.0) were used to cultivate the relative strains in this paper, respectively. The wild type strain *D. zeae* EC1 and its derived strains were shaked at 28 °C, and *Escherichia. coli* DH5α as indicator strain was shaked at 37 °C. Relative strains were cultivated with shaking at 100 rpm for phytotoxin detection; otherwise they were cultivated with shaking at 200 rpm. The following concentrations of antibiotics were used when required: ampicillin, 100 μg/mL; rifampicin, 50 μg/mL; kanamycin, 50 μg/mL; streptomycin, 40 μg/mL.

Transposon mutagenesis and In-frame deletion of *zmsO*

The transposon inserted mutant EM236 was obtained following the methods described previously (Zhou et al. 2011). The both flanking sequences of Tn5 were amplified and sequenced, and the ORF of *zmsO* was determined following the methods described previously (Cheng et al. 2013; Liu and Chen 2007).

In order to construct the deletion mutant of *zmsO*, from 458 bp upstream of *zmsO* to 85 bp downstream of starting site of *zmsO* was amplified as upstream flanking regions by *zmsO*-1 and *zmsO*-2 (online resource 1), and *zmsO*-3 and *zmsO*-4 (online resource 1) were used to amplify downstream flanking region from 44 bp to 662 bp downstream of *zmsO*. The two fragments were

Table 1 Strains and plasmid used in this study

Strain or plasmid	Sources or characteristics[a]	Source or reference
strains		
EC1	Wild type strain of *Dickeya zeae*, Rif^r	Lab collection
EM236	Tn5 mutant derived from EC1, defective in zeamine production	This study
Δ*zmsO*	A mutant with partial missing of *zmsO* derived from EC1, Rif^r	This study
Δ*zmsO*(*zmsO*)	A *zmsO* knockout mutant with wild type *zmsO* coding region at the downstream of *lac* promoter, Amp^r	This study
EC1(*zmsO*)	Wild type strain EC1 with wild type *zmsO* coding region at the downstream of *lac* promoter, Amp^r	This study
CC118	The host cells in *E. coli* of plasmid constructs derived from pKNG101	Lab collection
DH5α	The host cells in *E. coli* of plasmid constructs derived from pBBR1-MCS4	Lab collection
HB101(pRK2013)	*Thr leu thi recA hsdR hsdM pro*, Km^r	Lab collection
AC1	Wild type strain of *Bacillus subtilis*	Lab collection
PAO1	Wild type strain of *Pseudomonas aeruginosa*	Lab collection
plasmids		
pKNG101	Knockout vector, Str^r	Lab collection
pKNG-*zmsO*'	pKNG101 carries the fusing flank frames of deleted part of *zmsO*, Str^r	This study
pBBR1-MCS4	Expression vector contains a *lacZ* promoter, Amp^r	Lab collection
pBBR1-*zmsO*	pBBR1-MCS4 carries the coding region of *zmsK* at downstream of *lac* promoter, Amp^r	This study
pRL27	A plasmid carrying Tn5, Km^r	Lab collection

[a] Rif^r, Amp^r, Km^r, or Str^r = rifampicin, ampicillin, kanamycin, or streptomycin resistant

fused using primers *zmsO*-1 and *zmsO*-4, and inserted into the suicide plasmid pKNG101 to construct the recombinant deletion vector pKNG101-*zmsO*. Then the recombinant plasmid was transferred into the wild type strain EC1 by tri-parental mating with pRK2013. After recombination, conjugators were picked from the plate supplemented with streptomycin and re-streaked onto a new MM agar plate supplemented with streptomycin, incubated at 28 °C for 24 h, and washed down with 1 ml of MM medium, and spread on MM agar plate supplemented with 5 % sucrose. The original DNA fragment of *zmsO* was replaced by the fused fragment to remove a 678 bps fragment from EC1 genome. The colonies were checked using primers *zmsO*-F and *zmsO*-R. So the internal DNA fragment from 86th away from the start codon to the stop codon of *zmsO* was deleted and confirmed by DNA sequencing.

Complementation of *zmsO* deletion mutant

Fragment contains the 720 bps coding region of *zmsO* from 46 bp upstream of *zmsO* to 63 bp downstream of *zmsO* was amplified using primers C*zmsO-Hind*III and C*zmsO-Bam*HI (online resource 1) for the complementation, and the lower cases added in front was the restriction enzyme cutting sites. The 829 bps PCR products were digested with *Bam*HI and *Hin*dIII and then cloned into the high copy vector pBBR1-MCS4, which digested with the same enzymes. Then *zmsO* will be promoted by the *lacZ* promoter before MCS sites from pBBR1-MCS4 in recombinant plasmid. The recombinant plasmid pBBR1-*zmsO* was then transferred into DH5α by heat shock. Colonies were selected from LB plate supplemented with 100 μg/mL ampicillin by PCR reaction using primers MCS-F and MCS-R (online resource 1) on pBBR1-MCS4, and then recombinant plasmid with right size of PCR products were confirmed by DNA sequencing.

pBBR1-*ydcB* and pBBR1-*pcpS* were also constructed in the same way using relevant primers (online resource 1). The fragment from 1 bp upstream of *ydcB* to the last bp of this gene which contains the 366 bps encoding region of *ydcB* was amplified from *Bacillus subtilis* AC1. The exactly 729 bps encoding region of *pcpS* was amplified from *P. aeruginosa* PAO1. Both of these two genes were under control of the *lacZ* promoter before MCS sites on pBBR1-MCS4 as *zmsO*.

These resulting constructs, pBBR1-*zmsO*, pBBR1-*ydcB* and pBBR1-*pcpS* were transformed into the *zmsO* deleted mutant by tri-parental conjugation, respectively. Transformants were selected on MM plate contains 100 μg/mL ampicillin. PCR detection was performed by primers MCS-F and MCS-R to confirm the in *trans* expression.

Determination of antimicrobial activity

For quantitative comparison of the antimicrobial activity of different strains, these strains were cultivated in MM medium at 28 °C with a shaking at 100 rpm until the OD_{600} to 1.8, and then 20 μL of the supernatant of each culture was added into the wells on bioassay plate using *E. coli* DH5α as indicator strain. After inoculated at 37 °C overnight, inhibition zones can be seen clearly. 1 unit of zeamines is defined as the inhibition zone with 1 mm radius.

Virulence assay

EC1 and the *zmsO* derived strains were cultivated in LB medium at 28 °C with a shaking at 200 rpm until the OD_{600} got to 1.5, then liquid cultures were injected into the back side of the tobacco leaves by disposable syringes without needle until the area of internal wetness reached approximately 1 cm^2 as standard. Hypersensitive reaction was observed after the inoculated tobacco plant grown in greenhouse with enough sunshine for one to two days in vivo, and photos were taken using camera Canon A620.

The same liquid cultures in LB medium were used to detect the pathogenicity on potato. Potato slices were inoculated with 2 μL of the liquid cultures (OD_{600} = 1.5) at the centre and kept at 28 °C for about 24 h with three wet filter papers under them. Photos were taken after the bacterial ooze produced.

For measurement of rice seeds germination rate, the strains were cultivated in MM medium at 28 °C with a shaking at 200 rpm until the OD_{600} to 1.5, then 20 seeds of rice cultivar Texian 25 were added into 5 mL of different liquid cultures and incubated at room temperature for 5 h, respectively. The rice seeds were then washed three times with sterilized water and transferred onto three piece of filter paper, together with 5 mL sterilized water in sterilized petri dish, and incubated at 28 °C with 16 h light and 8 h dark cycles for 7 days. Rice seeds were treated with MM medium as a blank control.

Bioassay of cell wall degrading enzyme activities

EC1 and the *zmsO* derived strains were cultivated in LB medium at 28 °C with a shaking at 200 rpm until OD_{600} = 1.5, then 20 μL of different supernatant was added into the wells on each enzyme assay plates: pectate lyase (Pel), polygalacturonase (Peh), cellulose (Cel) and protease (Prt). The enzyme activities were calculated referring the methods published by Chatterjee and his colleagues (Chatterjee et al. 1995).

RNA extraction and RT-PCR

Each of *zmsO* derived strains at logarithmic phase were mixed well with 2 volumes of RNA protector and incubated for 5 min at room temperature, then collected by centrifugation at 4 °C for 12 min at 7000 rpm, and the total RNAs of each strains were extract by RNeasy mini kit (Qiagen). DNAs were digested by turbo DNA-free kit (Thermo Fisher). RNAs were used as templates to run PCR to confirm there is no DNA contamination in RNA samples. Then cDNAs were generated using SuperScript III First-Strand Synthesis SuperMix (invitrogen). 10 ng cDNAs were used to amplify the *rho* fragments as reference using *rho*-R-F/R (Takle et al. 2007). After template normalization, two pairs of special primers *zmsA*-R-F/R and *zmsR*-R-F/R were used to detect the transcriptional levels under same condition.

Preparation for zeamines crude extracts

Extraction and purification of zeamines crude extracts were conducted following the previously method (Liao et al. 2015). And crude extracts of zeamines were dissolved in water to the final concentration is 100 μg/mL.

Results

Identification of a transposon mutant with a decreased antimicrobial activity

Since zeamines will inhibit the *E. coli* growth by formation an inhibition zone on bioassay plate (Zhou et al. 2011; Cheng et al. 2013; Liao et al. 2014; Masschelein et al. 2013), we can use the same bioassay plate to compare the antimicrobial activity between different mutants by the size of their inhibition zones on plates. To further study the biosynthesis mechanisms of zeamines in *D. zeae*, a Tn5

insertion mutant library was screened and over 100 candidate mutants with a decreased antimicrobial activity were collected. Among these mutants, EM236 showed a significantly decreased antimicrobial activity compared to wild type strain EC1. The inserted gene was sequenced by using the hi-TAIL PCR method (Liu and Chen 2007), and designated as *zmsO*, which encodes a protein with 239 amino acids and sharing high identity to the phosphopantetheinyl transferase component of siderophore synthetase (Accession number WP_022632844.1) from *Dickeya solani*. Domain analysis showed that ZmsO contains a conserved domain of the ACPS 4′-phosphopantetheinyl transferase superfamily in the region from the 114th to 227th amino acid, suggesting that ZmsO is likely to be a 4′-phosphopantetheinyl transferase and plays a role in post-translational modification of the enzymes involved in zeamines biosynthesis.

zmsO is essential for production of zeamines in *D. zeae*

To preclude the possibility of polar effect of transposon insertion, we generated the marker-free in-frame deletion

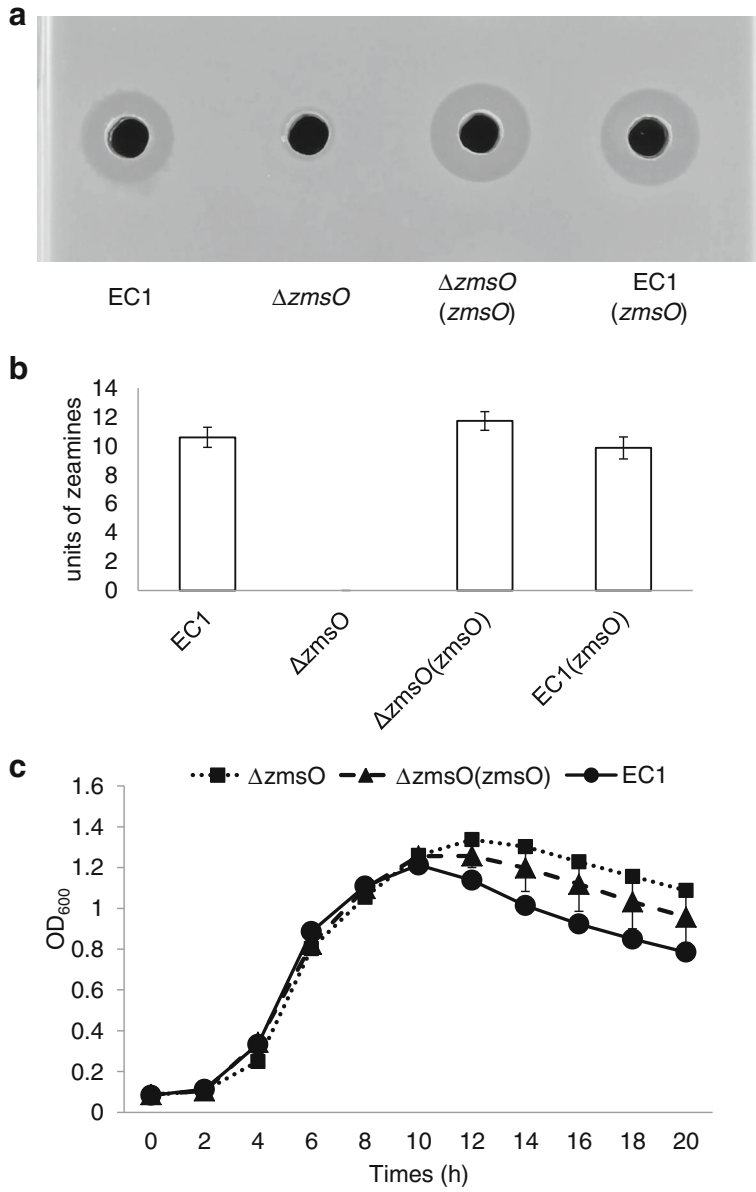

Fig. 1 Effect of *zmsO* on the antimicrobial activity (**a**) The same volume of supernatant of derived strains at the same cell density was spotted into the well on bioassay plates with *E. coli* cells as the indicator strain, after cultivation at 37 °C for overnight, *E. coli* cells can grow at the region without antimicrobial compounds. (**b**) The radius of each inhibition zones were measured, and calculated for the production units of zeamines. Error bars show the difference of three repeats. (**c**) Growth curve of wild type strain EC1, *ΔzmsO* and *ΔzmsO*(*zmsO*). And the error bars show the difference of three repeats

mutant of *zmsO* by deleting the DNA sequences encoding from the 29th to the end of its peptide product through homologous recombination. The mutant together with its wild type parental strain EC1 and complementary strains were assayed for zeamines production using *E. coli* DH5α, which is highly sensitive to zeamines (Wu et al. 2010; Zhou et al. 2011), as an indicator strain. Results showed that null mutation of *zmsO* completely abolished the zeamines production, while *in trans* expression of *zmsO* in the deletion mutant restored zeamines production to a level slightly higher than that of the wild type (Fig. 1b). We also tested whether overexpression of *zmsO* in wild type strain could enhance the yield of zeamines, but bioassay did not reveal any significant changes compared with strain EC1 (Fig. 1). Deletion of *zmsO* did not seem to affect bacterial growth, especially at the growth stages prior to stationary phase (Fig. 1c); suggesting the effect of ZmsO on zeamines production is not because the difference of growth rate.

Null mutation of *zmsO* attenuates bacterial virulence

In addition to its broad antibacterial activity (Wu et al. 2010), zeamine is also an important virulence factor of *D. zeae* (Zhou et al. 2011; Cheng et al. 2013). To test the impact of *zmsO* deletion on the bacterial virulence, we challenged tobacco leaves and potato tubes with the null mutant of *zmsO*, wild type strain EC1 and the complementary strain. Results showed that inoculation of EC1 and the complementary strain caused extensive localized cell death surrounding the inoculation site on tobacco leaves, whereas much smaller cell death zone was appeared on the leaves inoculated with the *zmsO* deletion mutant (Fig. 2a).

Similarly, the *zmsO* deletion mutant also showed attenuated virulence on potato tubers by showing smaller macerated infection zones than the wild type and complementary strains (Fig. 2b). In order to confirm if zeamines are the most key element to cause these pathogenicity changes, we also used the crude extract of zeamines to complement the *zmsO* deletion mutant. The scab on potato of this chemical complementation is a little bigger than that on mutant strain without complementation inoculated potato (online resource 2), which proved that zeamines can slightly restore the pathogenicity of *D. zeae* on potato. What's more, we found that deletion of *zmsO* did not affect the enzyme activities of polygalacturonase (Peh), pectate lyase (Pel), cellulose (Cel) and protease (Prt), which are tissue maceration enzymes and known important virulence factors of the bacterial pathogen (online resource 2), suggesting that zeamines may play a comparably important role in bacterial systemic infection and disease symptom development.

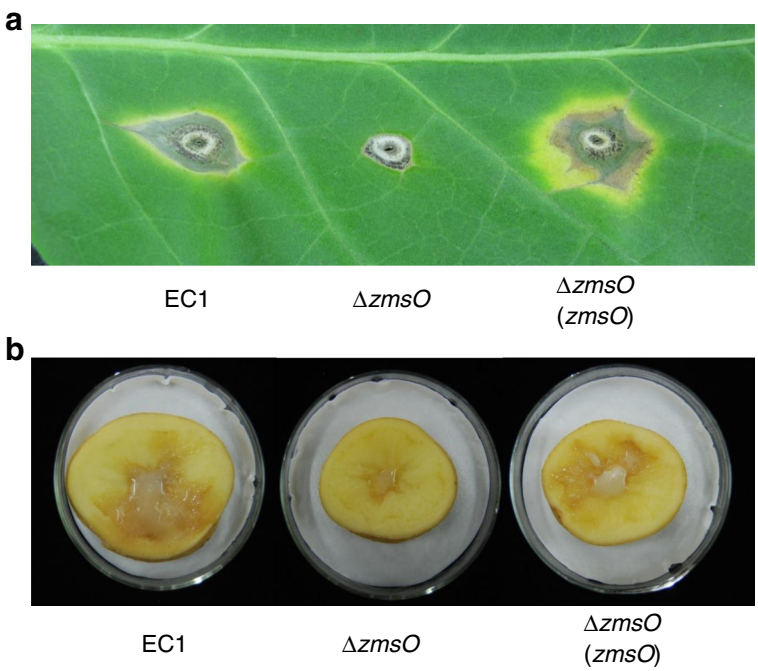

Fig. 2 Effect of *zmsO* on the pathogenicity (**a**) The same amount of cells of Δ*zmsO* and derived strains was injected into the mesophyll cell of tabacoo between leaf veins without artificial wounds. Scabs caused by different strains can be distinguished obviously after hypersensitive response occurred. (**b**) 2 μL of liquid cultures of derived strains at OD_{600} = 1.5 were injected onto the center of potato slices, rot scabs caused by null mutant of *zmsO* is much smaller than that of EC1 and complementary strain with less bacterial ooze. Each experiment has been repeated at least three times

Deletion of *zmsO* decreases the inhibitory activity of *D. zeae* on rice seeds germination

Our previous studies showed that both zeamine and zeamine II have the ability to inhibit the germination of rice seeds (Zhou et al. 2011), therefore, null mutation of ZmsO, which is critical for zeamines production, should attenuate the inhibitory activity of *D. zeae* on rice seeds germination. As expected, majority of the rice seeds soaked with blank MM medium (control) or the culture of the *zmsO* deletion mutant germinated well, in contrast, only a few rice seeds could germinate when treated with wild type strain EC1 and the complementary strain (Fig. 3a). We noted that the rice seeds treated with complementary strain had higher germination rate than wild type (Fig. 3a), which is most likely due to loss of the vector construct carrying *zmsO* during the 7-day period of incubation with rice seeds. It should be point out here that we defined seed germination as growth of either shoot or root or both. The rice seeds treated with the *zmsO* mutant grew both shoot and roots, while those treated with the complementary strain most grew shoot only (Fig. 3b). We have previously shown that the root germination of rice seeds is more sensitive to zeamines than shoot (Zhou et al. 2011). Taken together, these findings could explain the observed shoot germination in the rice seeds treated with complementary strain, which might produce less amount of zeamines than wild type as a portion of bacterial cells lost the complemented *zmsO*.

ZmsO is a Sfp-type PPTase, which affects the zeamines production at the post-translational modification level

Comparison of the identified genes associated with zeamines production in *D. zeae* to the zeamine

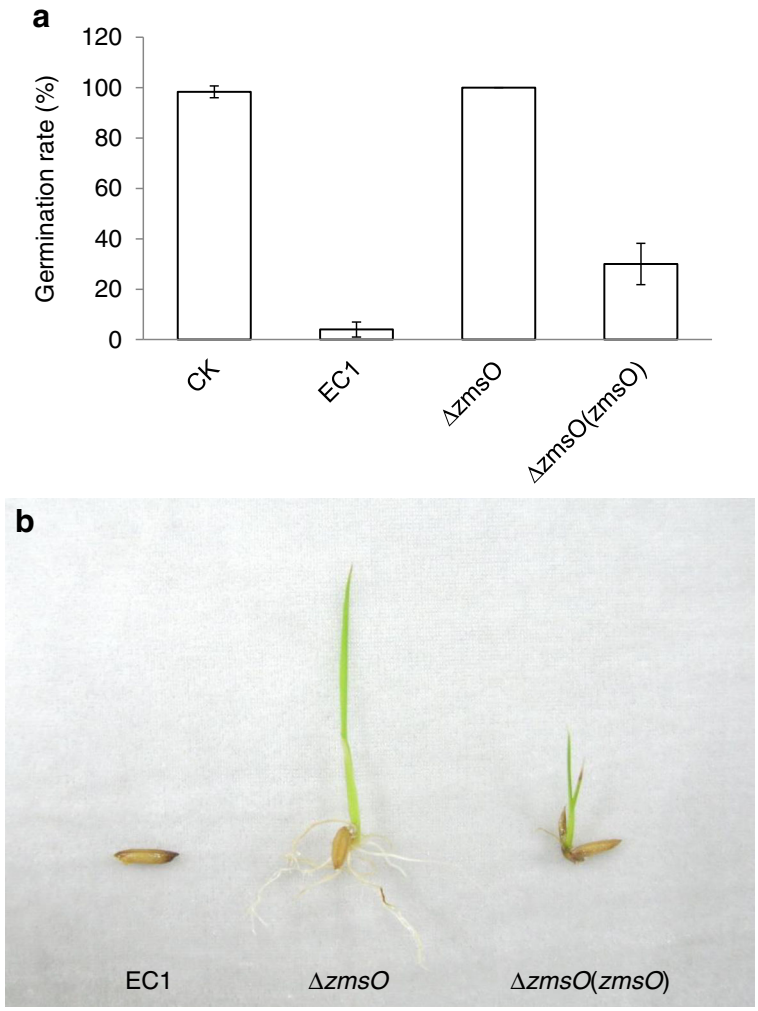

Fig. 3 Effect of *zmsO* on inhibition of rice seeds germination (**a**) The relative rice seeds germination rate of *D. zeae* wild type strain EC1 and derivatives. Blank MM medium was used as negative control. The experiment was repeated thrice with 20 seeds each time. The data shown are means of three times results with error bars indicating standard deviations. (**b**) Comparison of one representative seed soaked with derived strains after inoculation for 7 days

biosynthetic gene cluster in *S. plymuthica* RVH1 (Masschelein et al. 2013) showed that *zmsO* is at the upstream of the gene cluster and shares a 40 % identify with *zmn5* in *S. plymuthica* RVH1 at amino acids level (Fig. 4a). The *zms* gene cluster in *D. zeae* for zeamines biosynthesis has the similar structure with *zmn* gene cluster, and the corresponding genes share 50–92 % amino acids identity (Zhou et al. 2015). To further study the role of ZmsO in the production of zeamines, the transcriptional levels of two biosynthetic genes of zeamines in *zms* gene cluster, *zmsA* and *zmsK*, were measured and compared with wild type strain EC1 and *zmsO* derived strains. Results showed that the PCR product quantity of *zmsA* and *zmsK* are similar in wild type strain EC1 and *zmsO* derived strains (Fig. 4b). It proved that mutation of *zmsO* will not affect the transcriptional levels of polyketide synthase ZmsA (Zhou et al. 2011) and non-ribosomal peptide synthase ZmsK (Cheng et al. 2013) in *D. zeae*.

Analysis of the amino acid sequence of ZmsO showed that it is a 4′-phosphopantetheinyl transferase, which usually transform 4′-phosphopantetheine moiety from CoA to apo-CPs to form holo-CPs (Beld et al. 2014; Sunbul et al. 2009). Previous studies have classified the 4′-phosphopantetheinyl transferases into three types (Beld et al. 2014) (Fig. 5a). By analysis of the domain structures and conserved motifs, we predicted ZmsO as a Sfp-type PPTase as it contains three typical motifs P1, P2 and P3, while ACPS-type PPTase contains only P2 and P3 motifs (Fig. 5b). For further verification, we tested whether the *ydcB* gene from *Bacillus subtilis* (Mootz et al. 2001), which encodes an ACPS-type PPTase (AB001488), and the *pcpS* gene from *P. aeruginosa* (Finking et al. 2002), which encodes a Sfp-type PPTase, could functionally replace *zmsO*. The coding sequences of *ydcB* and *pcpS* were overexpressed under the control of *lacZ* promoter in the deletion mutant of *zmsO*. Sequence alignment showed that YdcB contains only P2 and P3 motifs, whereas PcpS and ZmsO contain P1-P3 three motifs (Fig. 5b). Bioassay results showed that overexpression of *ydcB* in Δ*zmsO* failed to restore the zeamines production (Fig. 6a). However, *in trans*

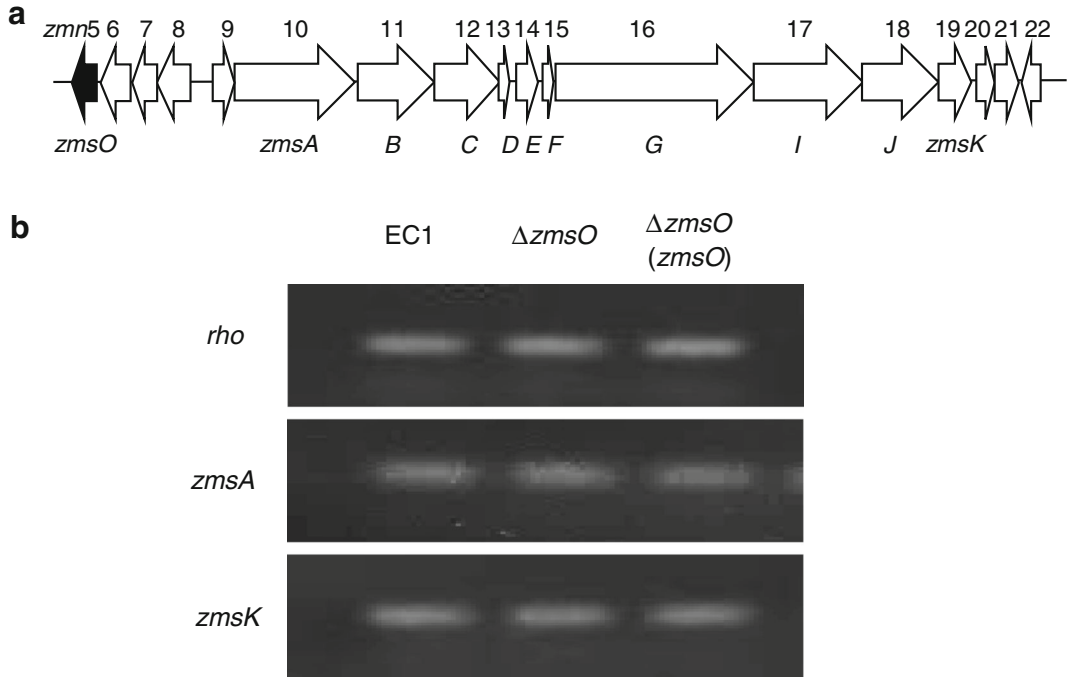

Fig. 4 Effect of *zmsO* on the transcription of two biosynthetic genes (**a**) The distribution of *zms* genes. *zmsO* (Accession number CP006929) is homologous gene of *zmn5* in zeamines biosynthetic gene cluster in *S. plymuthica* RVH1. Arrows indicate the sizes and transcriptional directions of each gene, labels above gene cluster in a line are the names in *S. plymuthica* RVH1, and bottom labels are homologous genes in *D. zeae*. (**b**) Effect of deletion of *zmsO* on transcriptional levels of *zmsA* and *zmsK*. Total cDNAs of *zmsO* derived strains were used as templates to amplifying of *zmsA* and *zmsK* with their special primers (online resource 1), respectively. *Rho* were used as reference genes

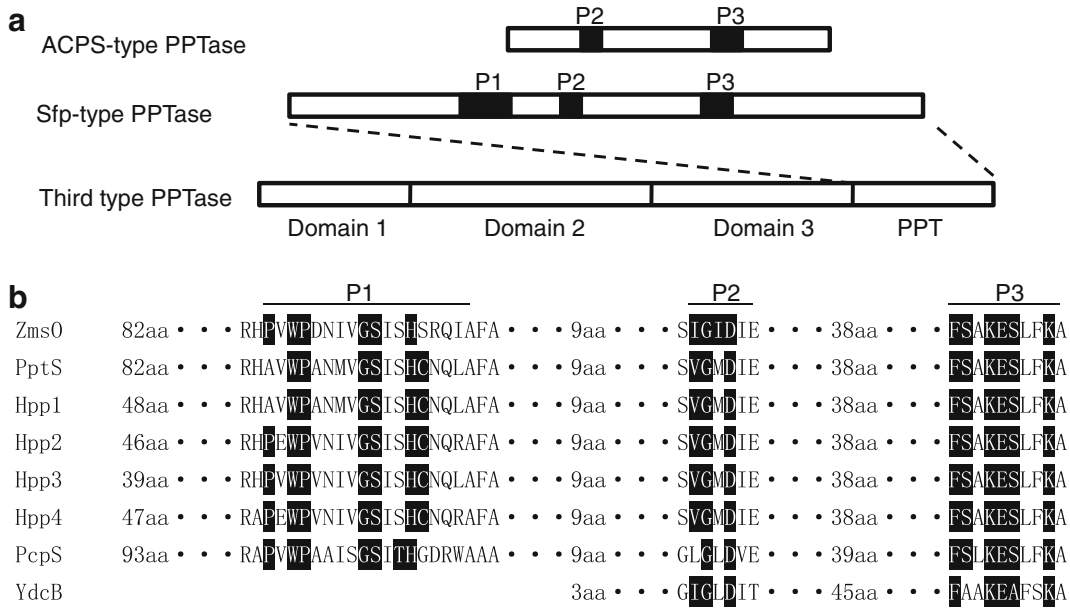

Fig. 5 Bioinformatics analysis of PPTases (**a**) Structure analysis of three types of PPTases. Three boxes in different lines indicate the complement ORF of three genes, the small *black* boxes inside represent the P1, P2 and P3 motif in PPTases. (**b**) Comparison of amino acid sequences of homologous proteins with ZmsO. PptS is the Phosphopantetheinyl transferase component of siderophore synthetase from *D. solani* D s0432–1 (ERO58214.1) which shares approximately 75 % identity in amino acids with ZmsO; Hpp1 is a hypothetical protein from *D. solani* (WP_035047690.1) which shares approximately 77 % identity in amino acids with ZmsO; Hpp2 is a hypothetical protein from *C. neteri* (WP_038668719.1) which shares approximately 79 % identity in amino acids with 72 %; Hpp3 is a hypothetical protein from *Dickeya.* sp. MK7 (WP_038920862.1) which shares approximately 79 % identity in amino acids; and Hpp4 is a hypothetical protein from *D. solani* (WP_039691492.1) which shares approximately 77 % identity in amino acids; PcpS (AAG04554) is Sfp-type PPTase from *P. aeruginosa* PAO1; YdcB (AB001488) is ACPS-type PPTase from *Bacillus subtilis* AC1. Shadowed letters show the amino acids corresponding to the conserved sites in motif

expression of *pcpS* in the *zmsO* deletion mutant completely rescued the zeamines production and virulence (Fig. 6). These results support the notion that ZmsO is a Sfp-type PPTase, which was required for the post-translational modification of zeamines.

Discussion

Zeamines are key virulence determinants of *D. zeae* (Zhou et al. 2011). These phytotoxins also showed broad effective antibiotic activities against numerous gram-positive and gram-negative bacteria, some fungal pathogens, and nematodes (Wu et al. 2010; Liao et al. 2015; Hellberg et al. 2015). To understand the biosynthesis pathway and regulatory mechanisms of zeamines in *D. zeae*, we used the transposon insertion mutagenesis methods to screen for the zeamines-deficient mutants and identified the *zmsO* gene encoding a putative 4′-phosphopantetheinyl transferase. Deletion of *zmsO* abolished the production of zeamines (Fig. 1) and substantially reduced the pathogenicity of *D. zeae* (Figs. 2, and 3). Overexpression of both the wild type *zmsO* and a Sfp-type PPTase encoding gene *pcpS* from *P. aeruginosa* PAO1 in the deletion mutant could restore zeamines production and pathogenicity of the mutant (Figs. 1, 2, 3, and 6). These results established that we have identified a Sfp-type PPTase ZmsO here which is essential for the production of zeamines through post-transcriptional modifing the secondary metabolite zeamines.

Null mutation of ZmsO abolished zeamines production, but did not seem to affect the bacterial growth rate, production of extracellular enzymes and transcriptional expression of zeamines biosynthesis genes *zmsA* and *zmsK* (Fig. 1, 4b, online resource 2). These findings are agreeable with the conserved roles of PPTase in post-translational activation of fatty acid synthases, polyketide synthases, and nonribosomal peptide synthetases (Liu et al. 2005; Murugan and Liang 2008; Orikasa et al. 2006); suggesting that the effect of ZmsO on zeamines production is mainly through modulation of the enzymes associated with zeamines biosynthesis.

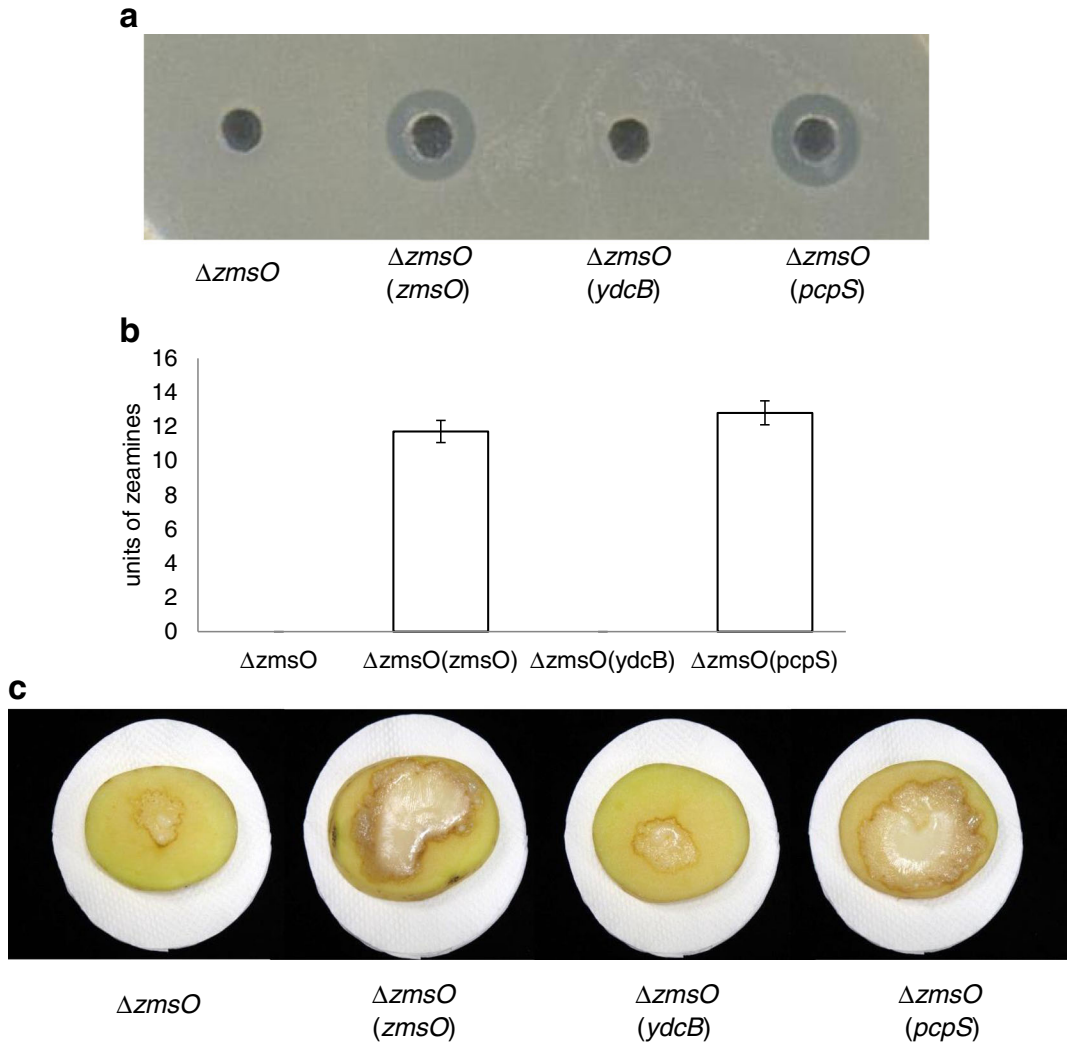

Fig. 6 Complementation of *zmsO* deleted mutant with different PPTase encoding genes Bioassay (**a**) and quantification (**b**) of zeamines production from deletion mutant of *zmsO*, complementary strains with *zmsO*, *ydcB* and *pcpS*, respectively. The corresponding units of zeamines were calculated based on the radius of inhibition zones. The data shown are the means of three repeats, and error bars indicate standard deviations. (**c**) Analysis of the pathogenicity of deletion mutant of *zmsO* complementing with *zmsO*, *ydcB* and *pcpS*, respectively

Since zeamines are the main virulence factor of *D. zeae* to cause the pathogenicity change between wild type strain EC1 and *zmsO* derived strains. Zeamines should have the ability to restore the pathogenicity of *zmsO* deletion mutant on potato. According to our previously study, EC1 can produce 50–80 μg/mL zeamines after 24 h incubation (unpublished), we used 100 μg/mL zeamines crude extracts to inoculate potato together with *zmsO* deletion mutant. But the results of crude extracts of zeamines to complement the *zmsO* deletion mutant on potato are not so well as the genetic complementation after 24 h (online resource 2). We guess that high concentration of zeamines will promote the immune response in plant as soon as they touched the plant cells, which started the immune response of potato much earlier than the potato slices inoculated with the wild type strain, deletion mutant of *zmsO* and complementary strain. So the chemical complementary inoculation will cause smaller scabs than genetic complementary inoculation with the same concentration of zeamines.

Recently, three zeamine derivatives including zeamine and zeamine II have also been found in *S. plymuthica* RVH1, and the biosynthesis gene cluster

has been reported (Masschelein et al. 2013; Masschelein et al. 2015). *D. zeae* EC1 contains a similar gene cluster with 18 ORFs (Fig. 4). Among them, *zmsA* has been characterized and reported to encode a polyketide synthase (Zhou et al. 2011), and *zmsK* is a nonribosomal peptide synthase encoding gene (Cheng et al. 2013), respectively. The transcriptional level of *zmsA* is lower in complementary strain than knock out mutant, but transcriptional level of *zmsK* is nearly the same in these two strains (Fig. 4). Mutation of *zmsA* abolished the production of both zeamine and zeamine II, while mutation of *zmsK* affects only zeamine (Zhou et al. 2011; Cheng et al. 2013). Given that mutation of either *zmsA* or *zmsO* leads to null production of zeamines, we are tempted to speculate that ZmsO may likely be involved in post-translational activation of ZmsA but not ZmsK. It is interesting to note that within the gene cluster for zeamines production, there are 5 ORFs encoding for polyketide synthases (Masschelein et al. 2013; Masschelein et al. 2015; Zhou et al. 2015). Obviously, further experiments are needed to identify the molecular target(s) of ZmsO.

The critical roles of PPTases in production of virulence factors and natural metabolites have been well documented. For example, PPTase is essential for the production of mupirocin in *P. fluorescens* (Shields et al. 2010), erythromycin in *Saccharopolyspora erythraea* (Weissman et al. 2004), fatty acid in *E. coli* strain O157:H7 (Lay and Lay and Cronan 2006), both fatty acid and siderophore in *P. aeruginosa* (Barekzi et al. 2004) and for the biosynthesis of virulence factors in Mycobacterium (Chalut et al. 2006). Identification of the role of ZmsO in zeamines production adds a new member to the list of PPTases which are associated with the biosynthesis of secondary metabolites with biological significances.

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