농학박사학위논문

벼흰잎마름병균이 분비하는 Type III Effector XopN과 벼의 단백질 OsVOZ2, Putative Thiamine Synthase의 상호작용

The Type III Effector XopN of *Xanthomonas oryzae* pv. *oryzae* Targets OsVOZ2 and a Putative Thiamine Synthase in Rice

2014년 2월

서울대학교 대학원

농생명공학부 식물미생물학전공

정 훈

The Type III Effector XopN of *Xanthomonas oryzae* pv. *oryzae* Targets OsVOZ2 and a Putative Thiamine Synthase in Rice

A dissertation submitted in partial
fulfillment of the requirement for
the degree of

DOCTOR OF PHILOSOPHY

to the Faculty of
Department of Agricultural Biotechnology

at

SEOUL NATIONAL UNIVERSITY

by

Hoon Cheong

February, 2014

농학박사학위논문

벼흰잎마름병균이 분비하는 Type III Effector XopN과 벼의 단백질 OsVOZ2, Putative Thiamine Synthase의 상호작용

지도교수 황 인 규

이 논문을 농학박사학위논문으로 제출함
2014년 2월

서울대학교 대학원
농생명공학부 식물미생물전공

정 훈

정 훈의 박사학위논문을 인준함
2014년 2월

위 원 장 _____

부위원장 _____

위 원 _____

위 원 _____

위 원 _____

A THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

The Type III Effector XopN of *Xanthomonas oryzae* pv. *oryzae* Targets OsVOZ2 and a Putative Thiamine Synthase in Rice

UNDER THE DIRECTION OF DR. INGYU HWANG

SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF SEOUL NATIONAL UNIVERSITY

BY
HOON CHEONG

MAJOR IN PLANT MICROBIOLOGY
DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY

FEBRUARY 2014

APPROVED AS A QUALIFIED THESIS OF HOON CHEONG
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
BY THE COMMITTEE MEMBERS

CHAIRMAN _____

VICE CHAIRMAN _____

MEMBER _____

MEMBER _____

MEMBER _____

The Type III Effector XopN of *Xanthomonas oryzae* pv. *oryzae* Targets OsVOZ2 and a Putative Thiamine Synthase in Rice

Hoon Cheong

ABSTRACT

Bacterial leaf blight on rice, one of the most serious rice diseases, is caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). To determine roles of *Xanthomonas* outer proteins (Xop) in *Xoo*, I identified seven Xop homologs from the genome information of *Xoo* KXO85. The $xopQ_{KXO85}$, $xopX_{KXO85}$, $xopP2_{KXO85}$, $xopP1_{KXO85}$, and $xopN_{KXO85}$ were generated by EZ-Tn*5* mutagenesis. Among the individual mutants, the $xopP2_{KXO85}$, $xopP1_{KXO85}$ and $xopN_{KXO85}$ mutants exhibited significantly reduced virulence compared to the wild-type in the flag leaves of Dongjin. Interestingly, I did not see reduced virulence when the $xopP2_{KXO85}$, $xopP1_{KXO85}$ and $xopN_{KXO85}$ mutants were

inoculated into young leaves of the Dongjin. This indicated that Xop-mediated virulence is dependent on leaf stages in rice. To identify target proteins of $XopN_{KXO85}$ in rice, I performed yeast two-hybrid screening. Two rice proteins, OsVOZ2 and putative thiamine synthase, appeared to interact with $XopN_{KXO85}$ in yeast. This interaction was further confirmed by bimolecular fluorescence complementation and *in vivo* pull-down assays. OsVOZ2 is a hypothetical protein, and OsXNP is a putative thiamine synthase. To investigate roles of OsVOZ2 in interactions between rice and *Xoo*, I evaluated virulence of the wild-type and the $xopN_{KXO85}$ mutant strain in the OsVOZ2 mutant rice generated by T-DNA insertion in Dongjin. Both the wild-type and the $xopN_{KXO85}$ mutant were less virulent in the mutant rice than in the wild-type rice. Therefore, $XopN_{KXO85}$ is a virulence factor that it plays an important role in the *Xoo* and rice interactions.

KEY WORDS: *Xanthomonas oryzae* pv. *oryzae*, *xop* genes, type III secretion system, yeast two-hybrid, bimolecular fluorescence complementation, pull-down assay

Student number: 2005-31098

This work was published: H Cheong, C-Y Kim, J-S Jeon, B-M Lee, J S Moon, I Hwang (2013) PLoS ONE 8(9): e73346. doi: 10.1371/journal.pone.0073346

CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

The Type III Effector XopN of *Xanthomonas oryzae* pv. *oryzae* Targets OsVOZ2 and a Putative Thiamine Synthase in Rice

ABSTRACT

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is spread systemically through the xylem tissue and causes bacterial blight in rice. I evaluated the roles of *Xanthomonas* outer proteins (Xop) in the *Xoo* strain KXO85 in a Japonica-type rice cultivar, Dongjin. Five *xop* gene knockout mutants ($xopQ_{KXO85}$, $xopX_{KXO85}$, $xopP1_{KXO85}$, $xopP2_{KXO85}$, and $xopN_{KXO85}$) were generated by EZ-Tn*5* mutagenesis, and their virulence was assessed in 3-month-old rice leaves. Among these mutants, the $xopN_{KXO85}$ mutant was less virulent than the wild-type KXO85. In contrast, the $xopP1_{KXO85}$, $xopP2_{KXO85}$, and $xopN_{KXO85}$ mutants exhibited significantly less virulence in flag leaves after flowering than the wild-type KXO85. These observations indicate that the roles of Xop in *Xoo* virulence are dependent on leaf stage. I chose the $xopN_{KXO85}$ gene for further characterization because the $xopN_{KXO85}$ mutant showed the greatest influence on virulence regardless of leaf stage. I confirmed that $XopN_{KXO85}$ is translocated into rice cells, and its gene expression is positively regulated by HrpX. Two rice proteins, OsVOZ2 and a putative thiamine synthase (OsXNP), were identified as targets of $XopN_{KXO85}$ by yeast two-hybrid screening. Interactions between $XopN_{KXO85}$ and OsVOZ2 and OsXNP were further confirmed *in planta* by

bimolecular fluorescence complementation and *in vivo* pull-down assays. To investigate the roles of OsVOZ2 in interactions between rice and *Xoo*, I evaluated the virulence of the wild-type KXO85 and $xopN_{KXO85}$ mutant in the *OsVOZ2* mutant line PFG_3A-07565 of Dongjin. The wild-type KXO85 and $xopN_{KXO85}$ mutant were significantly less virulent in the mutant rice line, and the bacterial population was decreased. These results indicate that $XopN_{KXO85}$ and OsVOZ2 play important roles both individually and together for *Xoo* virulence in rice.

INTRODUCTION

Xanthomonas oryzae pv. *oryzae* (*Xoo*) causes bacterial leaf blight, which is one of the most serious diseases in rice (*Oryza sativa* L.). This bacterium invades the xylem of rice leaves through hydathodes or wounds. The strain of *Xoo* KXO85 (KACC10331) was isolated from diseased rice leaves in Korea, and its whole genome sequence was published in 2005 (Lee *et al*., 2005).

Plant pathogenic bacteria belonging to the genera *Pseudomonas*, *Xanthomonas*, *Erwinia*, and *Ralstonia* possess the type III protein secretion system (T3SS) that is critical for full virulence and bacterial colonization in their host plants (Büttner *et al*., 2009; Büttner *et al*., 2002; Crabill *et al*., 2012; Gala'n and Collmer, 1999; Ghosh, 2004). The T3SS of plant pathogenic species of *Pseudomonas*, *Xanthomonas*, *Erwinia*, and *Ralstonia* is highly conserved and involved in translocation of T3SS-dependent effector proteins from bacterial cells into plant cells (Alfano and Collmer, 2004; Boureau *et al*., 2011; Göhre and Robatzek, 2008; Meyer *et al*., 2006; Mudgett, 2005; Petnicki-Ocwieja *et al*., 2005). These effector proteins are categorized into two groups: transcription activator-like (TAL) effectors and non-TAL effectors (Mudgett, 2005; Furutani *et al*., 2009; Scholze and Boch, 2011; White *et al*.,

2009). In *Xoo*, T3SS that is essential for virulence is encoded by hypersensitive response and pathogenicity (*hrp*) genes, the expression of which is controlled by HrpX (Furutani *et al*., 2009; Sugio *et al*., 2005; Zhu *et al*., 2000).

T3SS-dependent plant bacterial effectors are important for bacterial growth, colonization, virulence, and race specificity in their host plants (Bai *et al*., 2000; Boch and Bonas, 2010; Bogdanove *et al*., 2010; Hwang *et al*., 2012; Roden *et al*., 2004; Yu *et al*., 2011). However, the biochemical functions of most T3SS-dependent plant bacterial effectors in their hosts have not been well characterized. *Xanthomonas* outer proteins (Xop) are known as non-TAL bacterial effector proteins that are delivered to the plant cell via Hrp T3SS. The major roles of non-TAL bacterial effectors involve modulation of signaling in the plant defense response (Mudgett, 2005; Metz *et al*., 2005). For example, $XopX_{Xcv}$ from *X. campestris* pv. *vesicatoria* (*Xcv*) affects the virulence of *Xcv* on pepper (*Capsicum annuum*) and tomato (*Lycopersicum esculentum*) and targets basic innate immunity in plants (Metz *et al*., 2005). $XopD_{Xcv}$ is a small ubiquitin-like modifier (SUMO) protease in *Xcv* that promotes bacterial growth in tomato and slows leaf chlorosis and necrosis in tomato at late stages of infection (Kim *et al*., 2008). Another T3SS-dependent non-TAL effector, $XopN_{Xcc}$, plays important roles in colonization and virulence of *X. campestris* pv. *campestris* (*Xcc*) in their hosts (Jiang *et al*., 2008). XopN is highly

conserved among *Xanthomonas* species (Roden et al., 2004). In addition, $XopN_{Xcv}$ may suppress pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) in tomato (Kim *et al*., 2009).

Compared to known host targets of TAL effectors in xanthomonads, there have been few studies on non-TAL effector targets in xanthomonads. $XopD_{Xcv}$ may target nuclear SUMOylated proteins (Canonne *et al*., 2011). In *Xcc* and *Arabidopsis* interactions, $XopD_{Xcc}$ targets the transcription factor MYB30 to suppress host defense (Canonne *et al*., 2011). Recently, it was found that tomato transcription factor SlERF4 was identified as a target of $XopD_{Xcv}$ in tomato (Kim *et al*., 2013). Non-TAL effector Xoo1488 of *Xoo* MAFF311018 targets two receptor-like cytoplasmic kinases (RLCKs), Os01g0936100 (OsRLCK55) and Os05g0372100 (OsRLCK185), to inhibit OsRLCK185 phosphorylation and the downstream MAPK signaling (Yamaguchi *et al*., 2013). Other reported host targets of $XopN_{Xcv}$ are tomato atypical receptor-like kinase (TARK1) and four 14-3-3 isoforms (TFT1, TFT3, TFT5, and TFT6) (Kim *et al*., 2009).

In *Xoo*, considerable efforts have been made to characterize functional roles of TAL effectors in various strains (Gu *et al*., 2005; Verdier *et al*., 2012). The contribution of each TAL effector protein to *Xoo* virulence varies; some are critical for virulence, while others have relatively moderate roles (Gu

et al., 2005; Romer *et al*., 2010). However, the roles of non-TAL effectors in *Xoo* virulence have been poorly investigated. When 18 non-TAL effectors were evaluated for virulence in the Philippine strain PXO99^{A}, deletion of both copies of $xopZ_{PXO99}$ conferred significant reduction of virulence, whereas the other non-TAL effectors showed little influence on virulence in 4-week-old rice leaves (Song and Yang, 2010). Disease severity of *Xoo* in susceptible cultivars varies depending on leaf stage (Goel and Gupta, 1990; Mew, 1987; Sun *et al*., 2004; Zhang and Mew, 1985). This led us to assess the virulence of each *xop* mutant at the adult stage in the field with the expectation of more distinct and different disease response outcomes compared to virulence assay results at the young leaf stage. Here, I report the individual contributions of $XopN_{KXO85}$ to *Xoo* virulence in the Korean strain KXO85 at flag leaf stage in the field, identification of targets of $XopN_{Xoo}$ in rice, and their important roles for *Xoo* virulence.

MATERIALS AND METHODS

I. Bacterial strains

The bacterial strains and plasmids used in this study are listed in Table 1. All of the *Xoo* strains used were derivatives of the parent strain KXO85 (KACC10331). *Escherichia coli* cells were grown at 37°C in Luria–Bertani (LB) broth or on LB agar plates. The *Xoo* strains were grown at 28°C in PS broth (peptone 1%, sucrose 1%, sodium L-glutamate 0.1%) or PS agar (PSA) plates. Antibiotics were used at the following concentrations: ampicillin, 100 μg/mL; gentamycin, 20 μg/mL; kanamycin, 50 μg/mL; tetracycline, 10 μg/mL; and spectinomycin, 50 μg/mL for *E. coli* strains and cephalexine 10 μg/mL; gentamycin, 10 μg/mL; tetracycline, 2 μg/mL; and kanamycin, 25 μg/mL for *Xoo* strains.

II. Transposon insertion and marker-exchange mutagenesis

All recombinant DNA techniques were performed according to standard methods (Sambrook *et al*., 1997). To generate the $xopN_{KXO85}$ mutant, the approximately 3-kb *Bam*HI fragment carrying the $xopN_{KXO85}$ gene from BAC clone G2 (Table 1) of *Xoo* KXO85 was cloned into pML122. EZ-Tn*5*<TET-1> was inserted into the coding region of $xopN_{KXO85}$ in pML122

Table 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics[a)]	Reference or source
Xanthomonas oryzae* pv. *oryzae		
KXO85	KACC10331, wild-type, Korean race 1 strain that is virulent to rice carrying the *Xa21* resistance gene, $Ceph^R$	Lee *et al*., 2005
KXO85 $xopQ_{KXO85}$::EZ-Tn*5*	*xopQ* mutant of KXO85, Tet^R	This study
KXO85 $xopX_{KXO85}$::EZ-Tn*5*	*xopX* mutant of KXO85, Tet^R	This study
KXO85 $xopP1_{KXO85}$::EZ-Tn*5*	*xopP1* mutant of KXO85, Tet^R	This study
KXO85 $xopP1_{KXO85}$::EZ-Tn*5* (pML122B7)	*xopP1* mutant complemented with pML122G2, Tet^R, Gm^R	This study
KXO85 $xopP2_{KXO85}$::EZ-Tn*5*	*xopP2* mutant of KXO85, Tet^R	This study
KXO85 $xopP2_{KXO85}$::EZ-Tn*5* (pML122B7)	*xopP2* mutant complemented with pML122G2, Tet^R, Gm^R	This study
KXO85 $xopN_{KXO85}$::EZ-Tn*5*	*xopN* mutant of KXO85, Tet^R	This study
KXO85 $xopN_{KXO85}$::EZ-Tn*5* (pML122G2)	*xopN* mutant complemented with pML122G2, Tet^R, Gm^R	This study
KXO85 $hrpX_{KXO85}$::EZ-Tn*5*	*hrpX* mutant of KXO85, Km^R	This study
KXO85 $hrpB5_{KXO85}$::EZ-Tn*5*	Type III secretion system-deficient mutant of KXO85, Km^R	This study

Continued on following page

Table 1. - Continued

Escherichia coli		
DH5α	F$^-$ Φ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17* ($r_K^-m_K^+$) *deoR thi-1 supE44 λ-gyrA96 relA1*	Gibco BRL
DB3.1	F$^-$ gyr*A462* end*A1* Δ(*sr1*-rec*A*) mcr*B* mrr hsd*S20*(*rB-, Mb*-) sup*E44* ara-*14* gal*K2* lac*Y1* pro*A2* rps*L20*(Sp^R) xyl-*5 λ*- leu mtl*1*	Invitrogen
BAC clone		
G2	BAC plasmid containing XOO0279 to XOO0382 ORFs in *Xoo* KXO85 genome	Lee *et al*., 2005
Saccharomyces cerevisiae		
MaV203	MATα, *leu*2–3,112, *trp*1–901, *his*3Δ200, *ade*2–101, *gal*4Δ, *gal*80Δ, *SPAL*10::*URA*3, *GAL*1::*lacZ*, *HIS*3$_{UAS\ GAL1}$::*HIS*3@*LYS*2, *can*1^R, *cyh*2^R	Invitrogen
Agrobacterium tumefaciens		
C58C1 (pCH32)	Rif^R, Tet^R	Mudgett *et al*., 2000
Plasmids		
pML122	OriV, OriT, Gm^R, *p*Nm (*npt*II), broad host range expression vector	Labes *et al*., 1990
pXopN::EZ-Tn*5*	EZ-Tn*5*was inserted into the coding region of $xopN_{KXO85}$ in pML122	This study
pML122G2	pML122 containing the $xopN_{KXO85}$ coding sequence and flanking region cloned into *Bam*HI site	This study
pML122B7	pML122 containing the $xopP2_{KXO85}$ and $xopP1_{KXO85}$ coding sequence and flanking region cloned into *Hin*dIII site	This study

Continued on following page

Table 1. - Continued

pMLTC	Cya reporter gene was cloned into pML122, Gm^R	This study
pMCXopN	pMLTC containing the $xopN_{KXO85}$ coding sequence, Gm^R	This study
pDONR222	Gateway pDONR vector with pUC origin, T7 promoter/priming site, M13 forward (-20) and reverse priming sites; *rrnB* T1 and T2 transcription terminators, *att*P1 and *att*P2 sites, *ccd*B gene, Km^R, Cm^R	Invitrogen
pDONR-XopN	pDONR222 containing the $xopN_{KXO85}$ gene	This study
pDEST32	Gateway destination bait vector, GAL4 DNA binding domain (GAL4 DBD), *ccd*B gene, *LEU*2 gene, Cm^R, Gm^R	Invitrogen
pDEST22	Gateway destination prey vector, GAL4 activation domain (GAL4 AD), *ccd*B gene, *TRP1* gene, Cm^R, Amp^R	Invitrogen
pD32xopN	pDEST32 containing $xopN_{KXO85}$ coding sequence. Gm^R	This study
pD22Lib	pDEST22 containing the approximately 0.5–3 kb fragment of rice cDNA Library, Amp^R	This study
pD22OsVOZ2	pDEST22 containing the *OsVOZ2* coding sequence of rice, Amp^R	This study
pD22OsXNP	pDEST22 containing the *OsXNP* coding sequence of rice, Amp^R	This study
pENTR D TOPO	*att*L1 and *att*L2 sites for site-specific recombination of the entry clone with a gateway destination vector, Km^R	Invitrogen
pENTR-XopN	pENTR D TOPO harboring the $xopN_{KXO85}$ (without the stop codon) gene, Km^R	This study
pENTR-OsVOZ2	pENTR D TOPO harboring the *OsVOZ2* (without the stop codon) gene, Km^R	This study
pENTR-OsXNP	pENTR D TOPO harboring the OsXNP (without the stop codon) gene, KmR	This study

Continued on following page

Table 1. - Continued

pSY735 (735-YC)	Contains C-terminal (YC) fragments of the YFP protein vector, *att*R1 and *att*R2 sites for site-specific recombination of the destination clone with a gateway entry vector, Amp^R	Seo et al., 2011
pSY736 (736-YN)	Contains N-terminal (YN) fragments of the YFP protein vector, *att*R1 and *att*R2 sites for site-specific recombination of the destination clone with a gateway entry vector, Amp^R	Seo et al., 2011
pSY736-XopN	pENTR-XopN was recombined with pSY736 using gateway LR recombination	This study
pSY735-OsVOZ2	pENTR-OsVOZ2 was recombined with pSY735 using gateway LR recombination	This study
pDEST-SCYNE(R)^{GW}	The binary BiFC gateway destination vector, SCFP3A N-terminus, Flag linker, Km^R, Cm^R	Gehl *et al*., 2009
pDEST-SCYCE(R)^{GW}	Binary BiFC gateway destination vector, SCFP3A C-terminus, HA linker, Km^R, Cm^R	Gehl *et al*., 2009
pEXP-SCYNE(R)-Cnx7	*A. thaliana* gene Cnx7 was cloned into pDEST-SCYNE(R)^{GW} using gateway LR recombination.	Gehl *et al*., 2009
pEXP-SCYCE(R)-Cnx6	*A. thaliana* gene Cnx6 was cloned into pDEST-SCYCE(R)^{GW} using gateway LR recombination	Gehl *et al*., 2009
pSCYNE(R)-XopN	pENTR-XopN was recombined with pDEST-SCYNE(R)GW using gateway LR recombination	This study

Continued on following page

Table 1. - Continued

pSCYCE(R)-OsXNP	pENTR-OsXNP was recombined pDEST-SCYCE(R)^{GW} using gateway LR recombination	This study
p2GWF7	C-terminal GFP fusion, *att*R1 and *att*R2 sites for site-specific recombination of the destination clone with a gateway entry vector, Amp^{R}	Karimi *et al*., 2002
p2GWF7-XopN	pENTR-XopN was recombined with p2GWF7 using gateway LR recombination	This study
p2GWF7-OsVOZ2	pENTR-OsVOZ2 was recombined pDEST-SCYCE(R)^{GW} using gateway LR recombination	This study
pGWB5	Binary gateway vector, 35S promoter, C-terminal sGFP tag, *ccd*B gene, Km^{R}, Cm^{R}	Nakagawa *et al*., 2007
pGWB6	Binary gateway vector, 35S promoter, N-terminal sGFP tag, *ccd*B gene, Km^{R}, Cm^{R}	Nakagawa *et al*., 2007
pGWB5-OsXNP	pENTR-OsXNP was recombined pGWB5 using gateway LR recombination, Km^{R}	This study
pGWB8	Binary gateway vector, 35S promoter, C-terminal 6xHis epitopes tag, *ccd*B gene, Km^{R}, Cm^{R}	Nakagawa *et al*., 2007
pGWB8-XopN	pENTR-XopN was recombined with pGWB8 using gateway LR recombination, Km^{R}	This study
pGWB11	Binary gateway vector, C-terminal FLAG epitope tag, Km^{R}, Cm^{R}	Nakagawa *et al*., 2007

Continued on following page

Table 1. - Continued

pGWB11-OsXNP	pENTR-OsXNP was recombined pGWB11 using gateway LR recombination, Km^R	This study
pGWB12	Binary gateway vector, 35S promoter, N-terminal FLAG epitope tag, *ccd*B gene, Km^R, Cm^R	Nakagawa *et al*., 2007
pGWB12-OsVOZ2	pENTR-OsVOZ2 was recombined pGWB12 using gateway LR recombination, Km^R	This study

[a)] $Ceph^R$, cephalexin resistance; Gm^R, gentamycin resistance; Km^R, kanamycin resistance; Cm^R, chloramphenicol resistance; Sp^R, spectinomycin resistance; Tet^R, tetracycline resistance; Amp^R, ampicillin resistance; Nm, neomycin

by *in vitro* transposition according to the supplier's instructions (Epicentre) yielding pXopN::EZ-Tn*5* (Table 1). pXopN::EZ-Tn*5* was electroporated into *Xoo* KXO85, and the transformed cells were cultured on PSA medium containing tetracycline. The marker-exchanged mutant *Xoo* KXO85 $xopN_{KXO85}$::EZ-Tn*5* was isolated and confirmed by Southern hybridization. Transposon insertion and marker-exchange mutagenesis of the other *xop* genes ($xopQ_{KXO85}$, $xopX_{KXO85}$, $xopP2_{KXO85}$, and $xopP1_{KXO85}$) were performed by the same strategy as described above to generate the $xopN_{KXO85}$ mutant in *Xoo* KXO85.

III. Virulence assay

Rice plants of cultivar Dongjin were grown in a paddy field. The *OsVOZ2* mutant rice seeds (PFG_3A-07565; T_0 seed) were affirmed by the rice T-DNA Insertion Sequence Database (http://signal.salk.edu/cgi-bin/RiceGE) (Jeong *et al*., 2006). The homozygous T_7 transgenic mutant line of the *OsVOZ2* mutant rice was obtained and confirmed by RT-PCR analysis. Overnight cultures of *Xoo* cells were adjusted to approximately 1.8×10^8 CFU mL^{-1} and inoculated into 3-month-old leaves or fully expanded flag leaves by the scissor clip method (Kauffman *et al*., 1973). Symptoms were scored by measuring lesion lengths 21 days after inoculation. The growth of *Xoo* cells in plants was determined as described previously (Sugio *et al*., 2005).

IV. Quantitative real time RT-PCR analysis

The bacterial strains used were cultured in liquid medium XOM2 (Tsuge *et al*., 2002) or PSB for 24 h. Total RNA was isolated from the wild-type strain KXO85 and KXO85 *hrpX*$_{KXO85}$::EZ-Tn*5* using an RNeasy kit (Qiagen) according to the manufacturer's instructions. A total of 1 μg RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega) for 1 h at 42°C. RT-PCR products from samples were analyzed on agarose gels and the bacterial 16s rRNA was used as a standard. Quantitative real-time RT-PCR (qRT-PCR) was performed using the cDNA and gene-specific primers (Table 2). The transcription levels were determined by Power SYBR Green PCR Master Mix on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). The thermal cycling parameters were: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min. Expression of 16S rRNA was used to normalize the expression values in each sample, and relative expression values were determined against the average value of wild-type strain KXO85 using the comparative Ct method.

V. Adenylate cyclase assays

To generate the *xopN-cya* gene fusion protein, the *xopN*$_{KXO85}$ gene was cloned into the *Xba*I and *Xho*I sites of pMLTC to generate pMCXopN (Table 1)

Table 2. Primers used for qRT–PCR of *xopN*$_{KXO85}$.

Primer name[a]	Sequence (5′→3′)
XopN cDNA region	CACGGACAATACGCCCAGTTC
XopN (RT)-F	ATGAAACCTGCTGCATCCGC
XopN (RT)-R	CTCGGACTGTGCAGGCGAAT
16S rRNA cDNA region	TAAGTGAAGAGTTTGATCCTG
16S rRNA (RT)-F	GGCAGGCCTAACACATGCAA
16S rRNA (RT)-R	GTATTAGCGTAAGTTTCCCTAC

[a] F, forward primer; R, reverse primer.

followed by transformation into *Xoo* KXO85 and KXO85 *hrpB5*$_{KXO85}$::EZ-Tn*5*. For the assay of adenylate cyclase activity in rice leaf tissues, rice leaves were hand-inoculated with bacterial suspension using a needleless syringe. After 12 h, samples were frozen with liquid nitrogen and homogenized in assay buffer supplied with the cAMP Biotrak Enzyme Immunoassay System (GE Healthcare). The level of cAMP in leaf samples was measured by the cAMP Biotrak Enzyme Immunoassay System according to the manufacturer's directions.

VI. Yeast two-hybrid assay

A Gal4-based system with gateway technology (Invitrogen) was used for yeast two-hybrid assay. The *xopN*$_{KXO85}$ gene was amplified by PCR using *Xoo* KXO85 genomic DNA as a template. The PCR primers (Table 3) were flanked with attB1 and attB2 sites required for the gateway cloning system. The PCR product was cloned into the pDONR222 by BP recombination to generate the entry clone. Subsequently, the *xopN*$_{KXO85}$ gene was transferred into yeast destination bait plasmid pDEST32 by LR recombination resulting in pD32XopN (Table 1). To construct a Dongjin cDNA library, cDNA of approximately 0.5–3 kb was cloned into pDONR222 and subsequently into the prey plasmid pDEST22 by LR recombination yielding pD22Lib (Table 1). The pD32XopN has the DNA binding domain of Gal4 and the leucine selection marker gene *LEU2*. The pD22Lib has the

Table 3. Primers used for yeast two-hybrid system.

Primer name[a]	Sequence (5′→3′)
attB1xopN-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGAA ACCTGCTGCATCCGCC
attB2xopN-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTATTAC GCCGGCAGTGCCCG
Bait-F	AACCGAAGTGCGCCAAGTGTCTG
Bait and Prey-R	AGCCGACAACCTTGATTGGAGAC
Prey-F	TATAACGCGTTTGGAATCACT

[a] F, forward primer; R, reverse primer.

GAL4 transcription activation domain and the tryptophan selection marker gene *TRP1*. All constructs were checked by restriction enzyme analysis and confirmed by DNA sequencing. Both pD32XopN (bait) and pD22Lib (prey) plasmids were co-transformed into the yeast MaV203 strain according to the manufacturer's protocol (Invitrogen). The transformants were cultured on synthetic complete (SC) medium lacking leucine (–Leu) and tryptophan (–Trp). After 72 h, colonies were picked and mixed with 100 μL sterile water, and 10 μL cell suspension was spotted onto selection plates to screen for expression of the three reporter genes (*HIS3*, *URA3*, and *lacZ*). Growth of yeast transformants was assessed on SC –Leu –Trp –His supplemented with 0–50 mM 3-amino-1, 2, 4-triazole (3AT) as histidine inhibitor and SC –Leu –Trp –Ura. The change in the blue color of the transformants was monitored in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). I used the controls provided by Invitrogen: S (strong control: pEXP^{TM}32/Krev1 + pEXP^{TM}22/RalGDS-wt), W (weak control: pEXP^{TM}32/Krev1 + pEXP^{TM}22/RalGDS-m1), and A (absent control: pEXP^{TM}32/Krev1 + pEXP^{TM}22/RalGDS-m2).

VII. *Agrobacterium*-mediated transient expression

Agrobacterium infiltration into *N. benthamiana* leaves was performed as described previously (Kim *et al*., 2009). Cells of *A. tumefaciens* strain C58C1

(pCH32) (Mudgett *et al*., 2000) were cultured at 28°C for 2 days on LB agar medium containing 50 μg/mL kanamycin and 2.5 μg/mL tetracycline. The recombinant agrobacteria were grown in 10 mL LB liquid medium supplemented with appropriate antibiotics at 28°C and then harvested by centrifugation. The cell pellet was resuspended in buffer (10 mM MES, pH 5.6, 10 mM $MgCl_2$, and 150 μM acetosyringone), adjusted to a final OD_{600} of 0.6, and then incubated for 3 h at room temperature before inoculation. Cells were hand-infiltrated onto *N. benthamiana* leaves using a needleless 1 mL syringe. Inoculated plants were incubated at 26°C in a growth chamber for 1 to 2 days.

VIII. *In vivo* pull-down assay

To generate plasmids for Ni-NTA affinity pull-down assays, pENTR-XopN, pENTR-OsVOZ2, and pENTR-OsXNP were recombined into pGWB8, pGWB12, and pGWB11 by LR recombination yielding pGWB8-XopN, pGWB12-OsVOZ2, and pGWB11-OsXNP, respectively (Table 1). $XopN_{KXO85}$ was tagged with 6×His, OsVOZ2 was tagged with Flag at the N-terminal, and OsXNP was tagged with Flag at the C-terminal. The bacterial suspensions of *A. tumefaciens* coexpressing $XopN_{KXO85}$-6×His/Flag-OsVOZ2 and $XopN_{KXO85}$-6×His/OsXNP-Flag were hand-infiltrated into *N. benthamiana* leaves. At 30 h after infiltration, the leaves were frozen with liquid nitrogen and then

macerated in extraction buffer (100 mM sodium phosphate, pH 7.4, 20 mM imidazole, and 0.15% Triton X-100). Homogenized samples were mixed for 1 h at 4°C and centrifuged for 15 min at 17,000 × *g* at 4°C. The soluble extracts were incubated with 30 μL 50% slurry of Ni-NTA Superflow Agarose (Qiagen). Ni-NTA agarose was retrieved by centrifugation and washed three times with extraction buffer, and proteins were eluted with 8 M urea sample buffer followed by Western blotting analysis using anti-6×His (Qiagen) and anti-FLAG (Sigma) antibodies. Signals were visualized using detection solution (Immun-Star WesternC Kit; Bio-Rad).

IX. BiFC

The coding regions of *xopN*$_{KXO85}$, *OsVOZ2*, *OsXNP*, and *OsVOZ1* were amplified by PCR using proofreading DNA polymerase and appropriate primers (Table 4) and cloned into the Gateway entry vector pENTR D TOPO (Invitrogen) yielding pENTR-XopN, pENTR-OsVOZ2, pENTR-OsXNP, and pENTR-OsVOZ1, respectively. pENTR-XopN, pENTR-OsVOZ2, pENTR-OsXNP, and pENTR-OsVOZ1 were recombined into the Gateway binary BiFC vectors pDEST-SCYNE(R)^{GW} and pDEST-SCYCE(R)^{GW} using LR recombinase according to the manufacturer's instructions (Invitrogen) yielding pSCYNE-XopN, pSCYCE-OsVOZ2, pSCYCE-OsXNP, and pSCYCE-OsVOZ1,

Table 4. Primers used for BiFC, localization, and *in vivo* pull-down assay.

Primer name[a]	Sequence (5′→3′)
XopN (D-TOPO)-F	CACCATGAAACCTGCTGCATC
XopN (D-TOPO)-R	CGCCGGCGGCAGTGCCCGAT
OsVOZ2 (D-TOPO)-F	CACCATGGCCGGCGATCCG
OsVOZ2 (D-TOPO)-R	TGTCCCGTCACTAGGGTTC
OsXNP (D-TOPO)-F	CACCATGGCAGCCATGGCCA
OsXNP (D-TOPO)-R	GGCGTCCACGATCTCGCCGT

[a] F, forward primer; R, reverse primer.

respectively (Table 1). The constructs were confirmed by DNA sequencing and transformed into *A. tumefaciens* C58C1 (pCH32) for transient expression in *N. benthamiana* as described above. SCFP signals were detected using a confocal laser scanning microscope (Leica Microsystems) 26 h after infiltration.

X. Localization analysis of OsVOZ2, OsXNP and $XopN_{KXO85}$

To generate plasmids for sGFP visualization in *N. benthamiana*, pENTR-XopN, pENTR-OsVOZ2, and pENTR-OsXNP were recombined into pGWB5 by gateway LR to yield pGWB5-XopN, pGWB5-OsVOZ2, and pGWB5-OsXNP (Table 1). Cells of agrobacteria harboring pGWB5-XopN, pGWB5-OsVOZ2, and pGWB5-OsXNP were infiltrated into *N. benthamiana* leaves. Cells of *Agrobacterium* harboring pGWB6 or pGWB5 were used as positive or negative control, respectively. sGFP signals were observed using a fluorescence microscope (Carl Zeiss) 26 h after infiltration.

XI. Microscopy

I used confocal laser scanning microscopes (SCFP: TCS SP5; Leica Microsystems) to detect the SCFP signal, and sGFP fluorescence signals were detected with a fluorescence microscope (DE/Axio Imager A1 microscope; Carl Zeiss). The excitation and emission wavelengths were SCFP (458 nm and 465–480

nm, respectively) and sGFP (488 nm and 500–525 nm, respectively).

XII. Statistical analysis

JMP® 10 software (SAS Institute) was used for statistical analysis. Statistical significance was determined by a one-way or a two-way ANOVA with Tukey HSD post-test.

XIII. Ethics Statement

No specific permits were required for these kinds of field studies. This field is owned by the University Farm, College of Agriculture and life Sciences, Seoul National University. This university farm is located in Suwon, which is approximately 40 kilometers south of the main campus of Seoul National University in Seoul, Republic of Korea. The location is not privately-owned or protected in any way. The field studies did not involve endangered or protected species.

RESULTS

I. Mutagenesis of five *xop* genes in the Korean *Xoo* strain KXO85

Five *xop* genes, $xopQ_{KXO85}$ (XOO4466), $xopX_{KXO85}$ (XOO4287), $xopP1_{KXO85}$ (XOO3425), $xopP2_{KXO85}$ (XOO3426), and $xopN_{KXO85}$ (XOO0343) (Table 5) were characterized among 18 *xop* homologs in the strain KXO85 (www.xanthomonas.org/t3e.html), which showed significant homology with reported *xop* genes. EZ-Tn5 insertion mutants of $xopQ_{KXO85}$, $xopX_{KXO85}$, $xopP1_{KXO85}$, $xopP2_{KXO85}$, and $xopN_{KXO85}$ (Fig. 1) were generated in the strain KXO85, and then the virulence of each *xop* gene knockout mutant was evaluated in 3-month-old leaves of the Japonica-type rice cultivar Dongjin. Mutations in the $xopQ_{KXO85}$, $xopX_{KXO85}$, $xopP1_{KXO85}$, $xopP2_{KXO85}$, or $xopN_{KXO85}$ gene did not significantly affect virulence (Fig. 2A). When these five mutants were inoculated into the flag leaves of Dongjin in the field, the $xopP1_{KXO85}$, $xopP2_{KXO85}$, and $xopN_{KXO85}$ mutants were significantly less virulent than the wild-type KXO85 (Fig. 2B). Virulence of the $xopP1_{KXO85}$, $xopP2_{KXO85}$, and $xopN_{KXO85}$ mutants carrying each wild-type *xop* gene in a multicopy plasmid was recovered to the wild-type level (Fig. 2B). These observations indicate that $xopN_{KXO85}$ consistently exhibits important roles for virulence of *Xoo* regardless of leaf stage. Therefore, I chose

Table 5. Characteristics of five predicted *xop* genes from *Xoo* KXO85.

Gene	Locus in *X o o* KXO85	ORF size (bp)	a. a	M. W (Dalton)	pI	Conserved Domain Search by NCBI database	Homolog
xopQ	XOO4466	1395	464	50022.43	6.975	Nucleoside hydrolase (4)	HopQ1 (*P. syringae* pv. *phaseolicola*)
xopX	XOO4287	2109	702	74081.64	6.415	-	Hypothetical protein (*X. campestris* pv. *vesicatoria*)
xopP1	XOO3425	2133	710	77646.56	8.502	-	Hypothetical protein (*X. campestris* pv. *vesicatoria*)
xopP2	XOO3426	2142	713	77932.93	8.636	-	Hypothetical protein (*X. campestris* pv. *vesicatoria*)
xopN	XOO0343	2163	720	76020.12	8.445	-	HopAU1 (*P. syringae* pv. *phaseolicola*)

Fig. 1. Genetic organization of five *xop* genes and EZ-Tn*5* insertion positions in the *Xoo* KXO85 genome. The vertical bar with black open triangle indicates the position of the EZ-Tn*5* insertion. Arabic numerals on the left and right sides indicate the base position in the *Xoo* KXO85 genome.

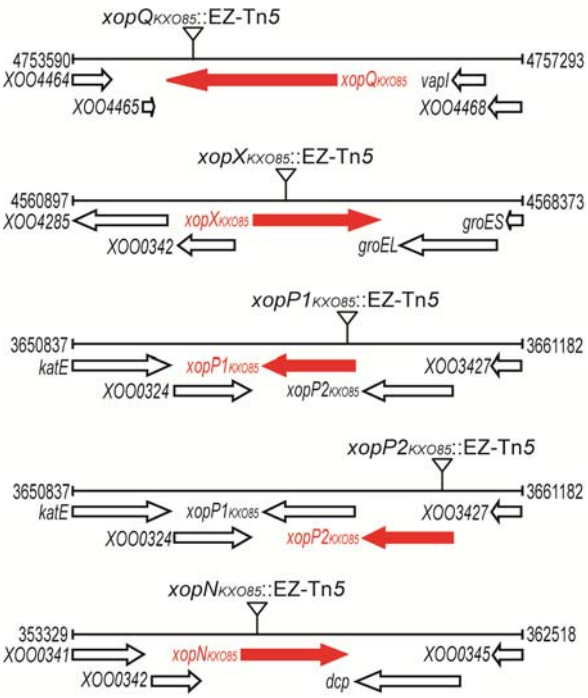
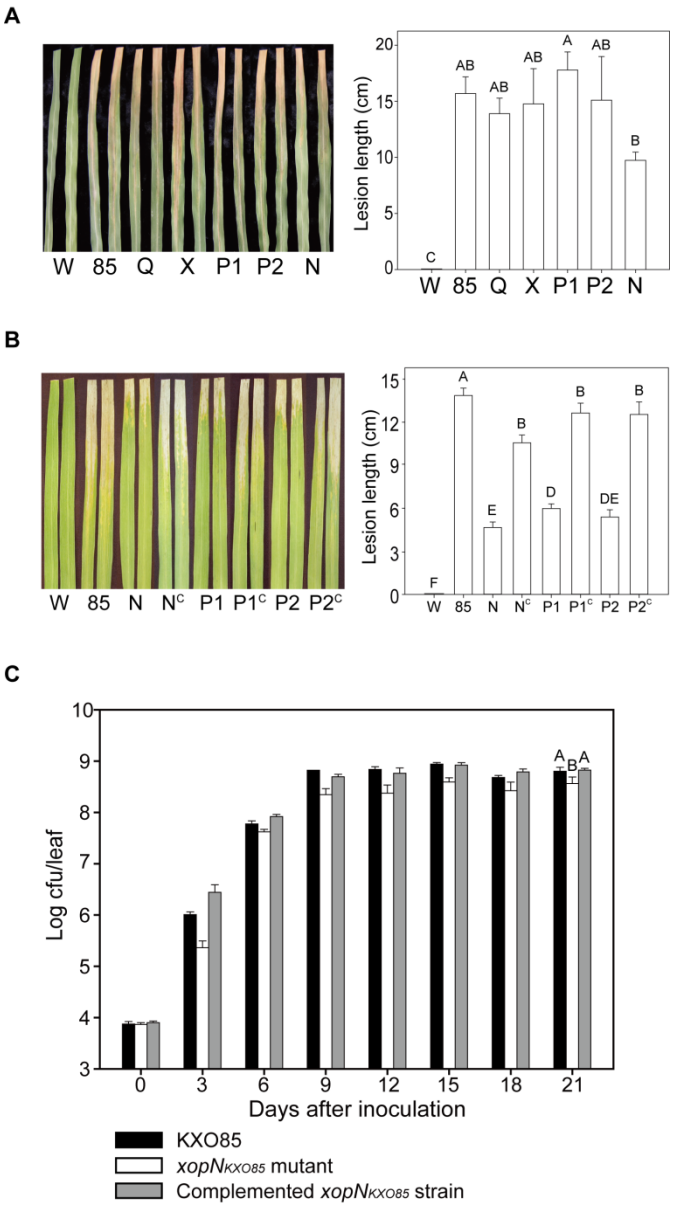

xopQKXO85::EZ-Tn5
4753590
4757293
XOO4464
xopQKXO85
vapI
XOO4465
XOO4468
xopXKXO85::EZ-Tn5
4560897
4568373
XOO4285
xopXKXO85
groES
XOO0342
groEL
xopP1KXO85::EZ-Tn5
3650837
3661182
katE
xopP1KXO85
XOO3427
XOO0324
xopP2KXO85
xopP2KXO85::EZ-Tn5
3650837
3661182
katE
xopP1KXO85
XOO3427
XOO0324
xopP2KXO85
xopNKXO85::EZ-Tn5
353329
362518
XOO0341
xopNKXO85
XOO0345
XOO0342
dcp

Fig. 2. Pathogenicity test for *xop* mutants of *Xoo* KXO85 in rice. (A) Disease severity of each *xop* mutant in 3-month-old rice leaves. W, Water; 85, wild-type KXO85; Q, KXO85 $xopQ_{KXO85}$::EZ-Tn*5*; X, KXO85 $xopX_{KXO85}$::EZ-Tn*5*; P1, KXO85 $xopP1_{KXO85}$::EZ-Tn*5*; P2, KXO85 $xopP2_{KXO85}$::EZ-Tn*5*; N, KXO85 $xopN_{KXO85}$::EZ-Tn*5*. (B) **.** Disease severity of the $xopP1_{KXO85}$, $xopP2_{KXO85}$, and $xopN_{KXO85}$ mutants in the flag leaves of rice grown in a paddy field. W, Water; 85, KXO85; N, KXO85 $xopN_{KXO85}$::EZ-Tn*5*; N^C, KXO85 $xopN_{KXO85}$::EZ-Tn*5* (pML122G2); P1, KXO85 $xopP1_{KXO85}$::EZ-Tn*5*; $P1^C$, KXO85 $xopP1_{KXO85}$::EZ-Tn*5* (pML122B7); P2, KXO85 $xopP2_{KXO85}$::EZ-Tn*5*; $P2^C$, KXO85 $xopP2_{KXO85}$::EZ-Tn*5* (pML122B7). Photographs were taken and lesion lengths were determined 21 days after inoculation. Vertical error bars indicate standard deviations (SD). Data are the averages of 12–15 replicates for each treatment. Columns and lines not connected with the same letter are statistically different ($p<0.05$) as determined by one-way ANOVA ($P<0.001$) followed by a post-hoc Tukey HSD analysis. (**C)** Bacterial growth patterns of KXO85, $xopN_{KXO85}$ mutant, and complemented $xopN_{KXO85}$ mutant strain in flag leaves in Dongjin. The data are shown as the average values for three replicates, and vertical bars indicate the error ranges (SD). Different letters at 21 day after inoculation indicate statically significant differences ($p<0.05$) as determined by one-way ANOVA ($P<0.001$) followed by a post-hoc Tukey HSD analysis.


A
W 85 Q X P1 P2 N
Lesion length (cm)
0 5 10 15 20
C AB AB AB A AB B
W 85 Q X P1 P2 N
B
W 85 N N^C P1 P1^C P2 P2^C
Lesion length (cm)
0 3 6 9 12 15
F A E B D B DE B
W 85 N N^C P1 P1^C P2 P2^C
C
Log cfu/leaf
3 4 5 6 7 8 9 10
0 3 6 9 12 15 18 21
Days after inoculation
A B A
KXO85
xopN KXO85 mutant
Complemented xopN KXO85 strain

xopN$_{KXO85}$ for further characterization. The bacterial population of the *xopN*$_{KXO85}$ mutant was reduced up to 21 days after inoculation compared to the growth of wild-type strain KXO85 in Dongjin (Fig. 2C).

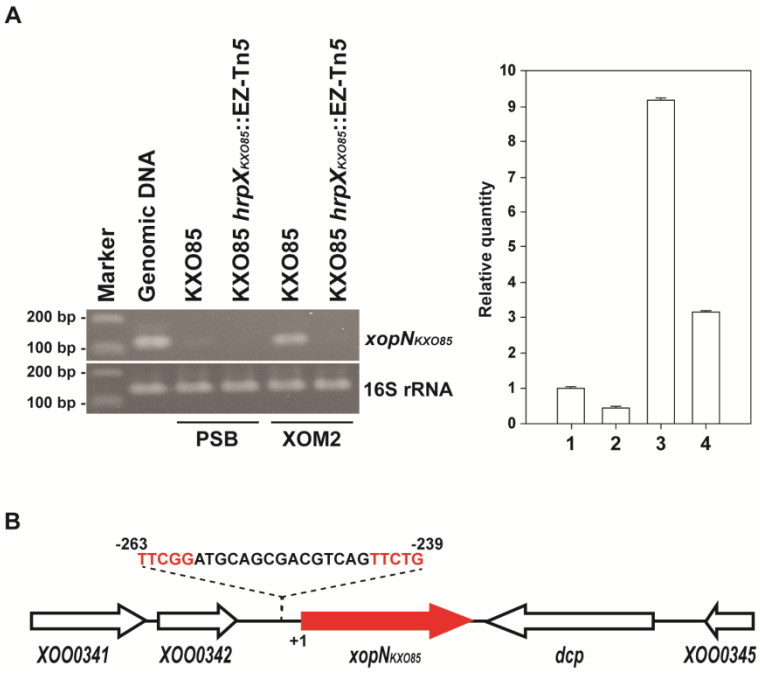
II. Expression of *xopN*$_{KXO85}$ is regulated by HrpX$_{KXO85}$

As expression of *hrp* and *xop* genes in *Xcv* and other xanthomonads is controlled by two regulatory genes, *hrpG* and *hrpX*, I examined whether *xopN*$_{KXO85}$ is regulated by HrpX$_{KXO85}$ in *Xoo* KXO85. Expression of *xopN*$_{KXO85}$ was below the limit of detection as assessed by quantitative real-time polymerase chain reactions (PCR) in the wild-type KXO85 or in the *hrpX*$_{KXO85}$ mutant strain in rich PSB medium (Fig. 3A). In the *hrp*-inducing medium XOM2, *xopN*$_{KXO85}$ expression in the wild-type KXO85 was approximately 3-fold higher than that in the *hrpX*$_{KXO85}$ mutant (Fig. 3A). I found a conserved *cis*-regulatory element plant-inducible promoter (PIP) box (TTCGG-N$_{15}$-TTCTG) in the region from –263 to –239 upstream of the start codon of *xopN*$_{KXO85}$ (Fig. 3B). These results indicate that *xopN*$_{KXO85}$ belongs to the HrpX$_{KXO85}$ regulon in *Xoo* KXO85.

III. XopN$_{KXO85}$ is a T3SS-dependent effector translocated into plant cells in the strain KXO85

To investigate whether XopN$_{KXO85}$ is translocated into plant cells in a

Fig. 3. Expression of *xopN*$_{KXO85}$ is regulated by HrpX$_{KXO85}$ in *Xoo* KXO85. (A) Expression profiles of *xopN*$_{KXO85}$ regulated by HrpX$_{KXO85}$ based on RT-PCR (left panel) and qRT-PCR (right panel) analyses. The 16S rRNA gene of KXO85 was used for normalization of cDNA quantity and expression value. Vertical error bars indicate standard deviations. 1, KXO85 grown in PSB; 2, KXO85 *hrpX*$_{KXO85}$::EZ-Tn5 incubated in PSB; 3, KXO85 grown in *hrp*-inducing medium XOM2; 4, KXO85 *hrpX*$_{KXO85}$::EZ-Tn5 incubated in XOM2. (B) The position of PIP box (TTCGG-N$_{15}$-TTCTG) is located near the *xopN*$_{KXO85}$ gene in the *Xoo* KXO85 genome.


A
Marker
Genomic DNA
KXO85
KXO85 hrpX_KXO85::EZ-Tn5
KXO85
KXO85 hrpX_KXO85::EZ-Tn5
200 bp
100 bp
200 bp
100 bp
xopN_KXO85
16S rRNA
PSB
XOM2
Relative quantity
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
B
-263
TTCGGATGCAGCGACGTCAGTTCTG
-239
+1
XOO0341
XOO0342
xopN_KXO85
dcp
XOO0345

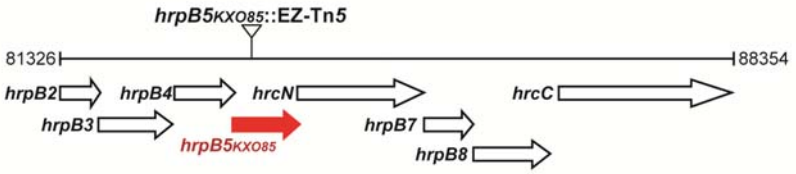
T3SS-dependent manner, I conducted a $XopN_{KXO85}$ translocation assay using the XopN-Cya fusion protein in the wild-type strain KXO85 and the T3SS-deficient mutant KXO85 *hrpB5*$_{KXO85}$::EZ-Tn*5* in rice (Fig. 4A). The level of cAMP increased in the wild-type strain KXO85, whereas no change in cAMP level was detected in the T3SS-deficient mutant KXO85 *hrpB5*$_{KXO85}$::EZ-Tn*5* (Fig. 4B). This indicates that $XopN_{KXO85}$ is translocated into rice cells in a T3SS-dependent manner.

IV. Identification of $XopN_{KXO85}$ targets in rice by yeast two-hybrid screening

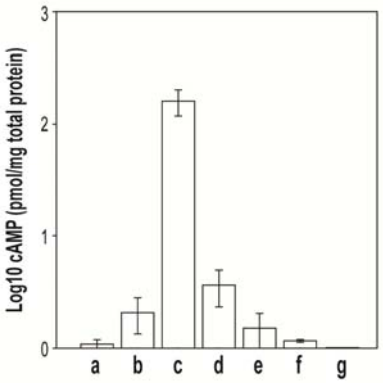
To identify $XopN_{KXO85}$ target proteins in rice, I carried out yeast two-hybrid screening using GAL4-$XopN_{KXO85}$ as a bait protein and a rice cDNA library constructed in the prey vector in the *Saccharomyces cerevisiae* strain MaV203. I found two possible candidates: *Oryza sativa* vascular plant one zinc finger protein 2 (OsVOZ2: NP_001056041, Os05g0515700) and *Oryza sativa* $XopN_{KXO85}$ binding protein (OsXNP: NP_001059841, Os07g0529600) (Fig. 5 and Table 6). The *OsVOZ2* gene is 3630 bp in length consisting of four exons and three introns and encodes a protein of 69901 Da. OsVOZ2 is a homolog of *Arabidopsis thaliana* vascular plant one zinc finger protein 2 (AtVOZ2; At2g42400) that has a conserved zinc finger domain (Fig. 6 and Fig. 7). The *OsXNP* gene is 1489 bp in length with two exons and one intron and possibly encodes a putative protein of

Fig. 4. Genetic map of the *hrpB5*$_{KXO85}$ mutant and cAMP assessment in rice leaves. (A) The vertical bar with a black open triangle indicates the position of the EZ-Tn*5* insertion in the *hrpB5*$_{KXO85}$ gene in the KXO85 genome. Arabic numerals on the left and right sides indicate the base positions in the KXO85 genome. (B) Levels of cAMP in rice leaves. a, KXO85; b, KXO85 *hrpB5*$_{KXO85}$::EZ-Tn5; c, KXO85 (pMC*xopN*); d, KXO85 *hrpB5*$_{KXO85}$::EZ-Tn*5* (pMC*xopN*); e, KXO85 (pMLTC); f, KXO85 *hrpB5*$_{KXO85}$::EZ-Tn*5* (pMLTC); g, Water. Each data point for all cAMP assays represents the average of three replicate samples with error bars indicating the standard deviation.

A



B



		cAMP (pmol/mg total protein)
Wild type	KXO85	1.09 ± 0.10
	T3SS deficient mutant	2.07 ± 0.73
*xopN*KXO85	KXO85	160.67 ± 42.18
	T3SS deficient mutant	3.63 ± 1.30
Vector control	KXO85	1.50 ± 0.53
	T3SS deficient mutant	1.16 ± 0.03
Water		0.62 ± 0.20

Fig. 5. Screening for interactors of $XopN_{KXO85}$ in rice by the yeast two-hybrid system. S (strong), W (weak), and A (absent) indicate strength of interactions. Three independent and representative colonies are shown for each bait–prey combination.

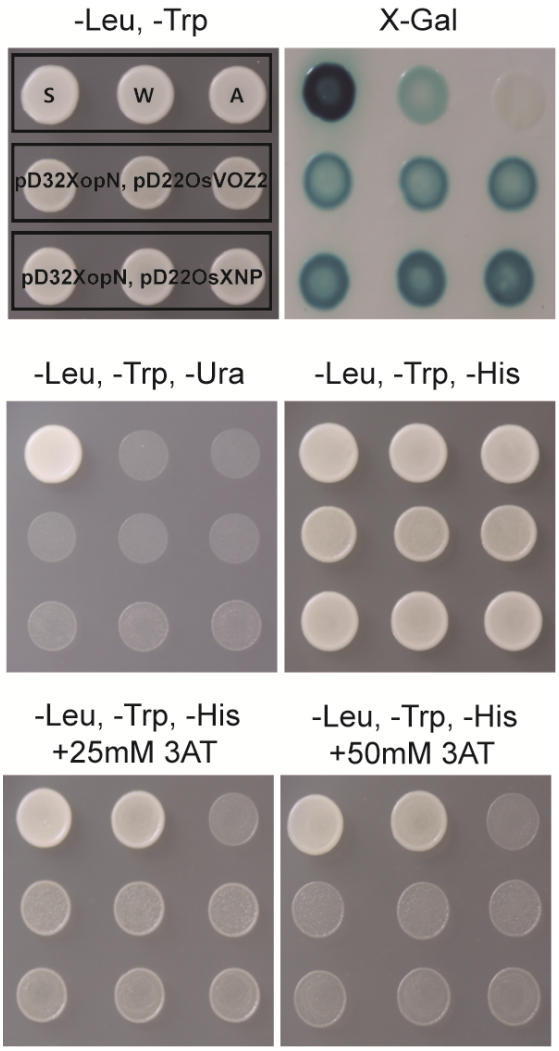

-Leu, -Trp
X-Gal
S
W
A
pD32XopN, pD22OsVOZ2
pD32XopN, pD22OsXNP
-Leu, -Trp, -Ura
-Leu, -Trp, -His
-Leu, -Trp, -His
+25mM 3AT
-Leu, -Trp, -His
+50mM 3AT

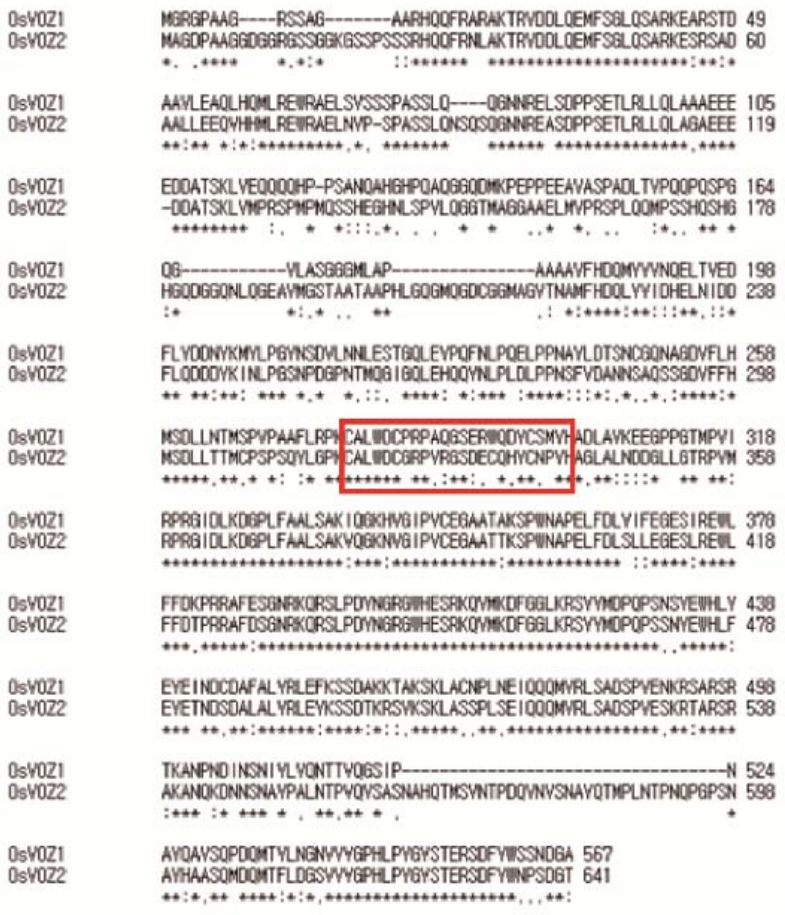
Table 6. Identification of OsVOZ2 and OsXNP.

Gene	Accession No.	Gene ID	Gene product	Homolog (GenBank Accession No.)
OsVOZ2	NP_001056041.1	Os05g0515700 [*Oryza sativa* Japonica Group]	Hypothetical protein	VOZ2 (AEC10117.1)
OsXNP	NP_001059841	Os07g0529600 [*Oryza sativa* Japonica Group]	Putative thiamine biosynthesis protein	thiamine biosynthetic enzyme (AFO59577.1)

Fig. 6. The amino acid sequence alignment of *A. thaliana* VOZ2 and OsVOZ2 using the Clustalw2 multiple alignment program.

```
AtVOZ2          MSNHP-------------KITSAHQNVEEKLR--------ELQERFCHLQAARKEGRHGD 39
OsVOZ2          MAGDPAAGGDGGRGSSGGKGSSPSSSRHQQFRNLAKTRVDDLQEMFSGLQSARKESRSAD 60
                *:.,*             * :*. .. .:::*        :*** *. **:****.* .*

AtVOZ2          LALLEAQISQNIREWQAELTAPSPESSLLG---EG----ISQFLEEFAPLLKLD---EED 89
OsVOZ2          AALLEEQVHHMLREWRAELNVPSPASSLQNSQSQGNNREASDPPSETLRLLQLAGAEEED 120
                 **** *: : :***:***,,*** *** ,   :*     *:  ,*   **:*    ***

AtVOZ2          DATSTLKEHAGAKPDP---EGFSQS-----------------------LCPPEWTSENFS 123
OsVOZ2          DATSKLVMPRSPMPMQSSHEGHNLSPVLQGGTMAGGAAELMVPRSPLQQMPSSHQSHGHG 180
                ****.*    .. *     **.. *                          *..  *....

AtVOZ2          Q-------------------SPFNGN---FSCGFEDALNSTETHGQQLHYGYEGFDPSIN 161
OsVOZ2          QDGGQNLQGEAVMGSTAATAAPHLGQGMQGDCGGMAGVTNAMFHDQLYYIDHELNIDDFL 240
                *                   :*. *:    .**   .:.,:  *,*  : .:*    .:

AtVOZ2          SAPDFHDQKLSSNLDITSQYDVI----FSEVRQELDNSPSTKLDSSEEIDN----FAEFS 213
OsVOZ2          QDDDVKINLPGSNPDGPNTMQGIGQLEHQQYNLPLDLPPNSFVDANNSAQSSGDVFFHMS 300
                .  *:: :  .** * ..  : *    ..: .  ** .*.: :*:.:. :.    * .:*

AtVOZ2          TPSSVRVP-PSAFLGPKCALWDCTRPAQGSEWYLDYCSNYHGTLALNED-SPGTAPVLRP 271
OsVOZ2          DLLTTMCPSPSQVLGPKCALWDCGRPVRGSDECQHYCNPYHAGLALNDDGLLGTRPVMRP 360
                   :.  * ** :********** **.:**:   .**. **. ****:*   ** **:**

AtVOZ2          GGISLKDNLLIDALRAKTQGKNVGIPVCEGAVNTKCPWNAAELFHLELVEGETIREWLFF 331
OsVOZ2          RGIDLKDGPLFAALSAKVQGKNVGIPVCEGAATTKSPWNAPELFDLSLLEGESLREWLFF 420
                 **.***. *: ** **.**************,.**.****.***.*.*:***::******

AtVOZ2          DKPRRAYDSGNRKQRSLPDYSGRGWHESRKQLMKEQEGQKRSYYMDPQPPGPFEWHLFEV 391
OsVOZ2          DTPRRAFDSGNRKQRSLPDYNGRGWHESRKQVMKDFGGLKRSYYMDPQPSSNYEWHLFEV 480
                *.****:*************.**********:**:  * **********,. :*******

AtVOZ2          QINESDACALYRLELKVGNGKKSPKGKISKDPLADLQKKMGQFKVASDKPSPPTKGRKE- 450
OsVOZ2          ETNDSDALALYRLEVKSSDTKRSVKSKLASSPLSEIQQQMVRLSADSPVESKRTARSRAK 540
                : *:*** ****** * .: *:* *.*::.,**:::*::* ::., *   *  *   :

AtVOZ2          ------------------------------------------------------------
OsVOZ2          ANQKDNNSNAYPALNTPVQVSASNAHQTMSVNTPDQVNVSNAVQTMPLNTPNQPGPSNAV 600


AtVOZ2          ----------------------------------------
OsVOZ2          HAASQMDQMTFLDGSVVYGPHLPYGYSTERSDFYWNPSDGT 641
```

Fig. 7. The amino acid sequence alignment of OsVOZ1 and OsVOZ2 using the Clustalw2 multiple alignment program. The red box represents conserved residues possibly forming a functional zinc-coordinating motif.

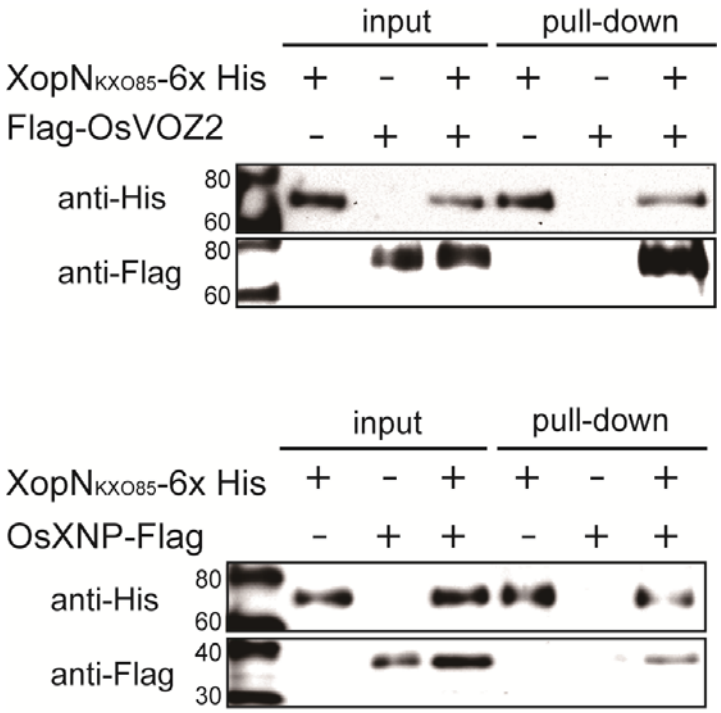

OsVOZ1 MGRGPAAG-----RSSAG--------AARHQQFRARAKTRVDDLQEMFSGLQSARKEARSTD 49
OsVOZ2 MAGDPAAGGDGGRGSSGGKGSSPSSSRHQQFRNLAKTRVDDLQEMFSGLQSARKESRSAD 60
OsVOZ1 AAVLEAQLHQMLREWRAELSVSSSPASSLQ-----QGNNRELSDPPSETLRLLQLAAAEEE 105
OsVOZ2 AALLEEQVHHMLREWRAELNVP-SPASSLQNSQSQGNNREASDPPSETLRLLQLAGAEEE 119
OsVOZ1 EDDATSKLVEQQQQHP-PSANQAHGHPQAQGGQDMKPEPPEEAVASPADLTVPQQPQSPG 164
OsVOZ2 -DDATSKLVMPRSPMPMQSSHEGHNLSPVLQGGTMAGGAAELMVPRSPLQQMPSSHQSHG 178
OsVOZ1 QG----------VLASGGGMLAP----------AAAAVFHDQMYYVNQELTVED 198
OsVOZ2 HGQDGGQNLQGEAVMGSTAATAAPHLGQGMQGDCGGMAGVTNAMFHDQLYYIDHELNIDD 238
OsVOZ1 FLYDDNVKMYLPGYNSDVLNNLESTGQLEVPQFNLPQELPPNAYLDTSNCGQNAGDVFLH 258
OsVOZ2 FLQDDDYKINLPGSNPDGPNTMQGIGQLEHQQYNLPLDLPPNSFVDANNSAQSSGDVFFH 298
OsVOZ1 MSDLLNTMSPVPAAFLRPMCALWDCPRPAQGSERWQDYCSMVHADLAVKEEGPPGTMPVI 318
OsVOZ2 MSDLLTTMCPSPSQYLGPMCALWDCGRPVRGSDECQHYCNPVHAGLALNDDGLLGTRPVM 358
OsVOZ1 RPRGIDLKDGPLFAALSAKIQGKHVGIPVCEGAATAKSPWNAPELFDLYIFEGESIREWL 378
OsVOZ2 RPRGIDLKDGPLFAALSAKVQGKNVGIPVCEGAATTKSPWNAPELFDLSLLEGESLREWL 418
OsVOZ1 FFDKPRRAFESGNRKQRSLPDYNGRGWHESRKQVMKDFGGLKRSYYMDPQPSNSYEWHLY 438
OsVOZ2 FFDTPRRAFDSGNRKQRSLPDYNGRGWHESRKQVMKDFGGLKRSYYMDPQPSSNYEWHLF 478
OsVOZ1 EYEINDCDAFALYRLEFKSSDAKKTAKSKLACNPLNEIQQQMVRLSADSPVENKRSARSR 498
OsVOZ2 EVETNDSDALALYRLEYKSSDTKRSVKSKLASSPLSEIQQQMVRLSADSPVESKRTARSR 538
OsVOZ1 TKANPNDINSNIYLVQNTTVQGSIP----------N 524
OsVOZ2 AKANQKDNNSNAVPALNTPVQVSASNAHQTMSVNTPDQVNVSNAVQTMPLNTPNQPGPSN 598
OsVOZ1 AYQAVSQPDQMTYLNGNVVYGPHLPYGYSTERSDFYWSSNDGA 567
OsVOZ2 AYHAASQMDQMTFLDGSVVYGPHLPYGYSTERSDFYWNPSDGT 641

37224 Da that has significant homology with thiamine biosynthetic enzyme in *Saccharum* hybrid cultivar GT28 (Table 3).

V. $XopN_{KXO85}$ physically interacts with two rice proteins OsVOZ2 and OsXNP *in planta*

To confirm the specific interactions between $XopN_{KXO85}$ and OsVOZ2 and $XopN_{KXO85}$ and OsXNP *in planta*, I performed affinity pull-down experiments in *N. benthamiana* leaves. Cells of *Agrobacterium tumefaciens* strain C58C1 (pCH32) carrying pGWB8-XopN (*xopN*$_{KXO85}$-6×His in pGWB8) or pGWB12-OsVOZ2 (*OsVOZ2*-Flag in pGWB12) were co-infiltrated into *N. benthamiana* leaves. For pull-down experiments to investigate interactions between $XopN_{KXO85}$ and OsXNP, *A. tumefaciens* cells harboring pGWB8-XopN and pGWB11-OsXNP (*OsXNP*-Flag in pGWB11) were co-infiltrated into tobacco leaves. Eluted soluble proteins bound to Ni-nitrilotriacetic acid (Ni-NTA) superflow agarose slurry were subjected to immunoblotting analysis using anti-His or anti-Flag antibodies. Both Flag-OsVOZ2 and OsXNP-Flag proteins were pulled down by $XopN_{KXO85}$-6xHis (Fig. 8). These results indicate that $XopN_{KXO85}$ physically interacts with OsVOZ2 or OsXNP in *N. benthamiana* leaves.

Fig. 8. *In vivo* pull-down analysis of $XopN_{KXO85}$ and OsVOZ2 (upper panel) and $XopN_{KXO85}$ and OsXNP (lower panel). Total proteins from tobacco leaves coexpressing $XopN_{KXO85}$-6×His and Flag-OsVOZ2 or $XopN_{KXO85}$-6×His and OsXNP-Flag protein were purified by Ni^{+} affinity chromatography followed by Western blotting analysis using anti-His and anti-Flag. Expected protein molecular weights are as follows: $XopN_{KXO85}$-6×His = 78.7 kD; Flag-OsVOZ2 = 74.6 kD; OsXNP-Flag = 40.1 kD; +, protein expressed; –, vector control.


input
pull-down
XopNKXO85-6x His + - + + - +
Flag-OsVOZ2 - + + - + +
anti-His
80
60
anti-Flag
80
60
input
pull-down
XopNKXO85-6x His + - + + - +
OsXNP-Flag - + + - + +
anti-His
80
60
anti-Flag
40
30

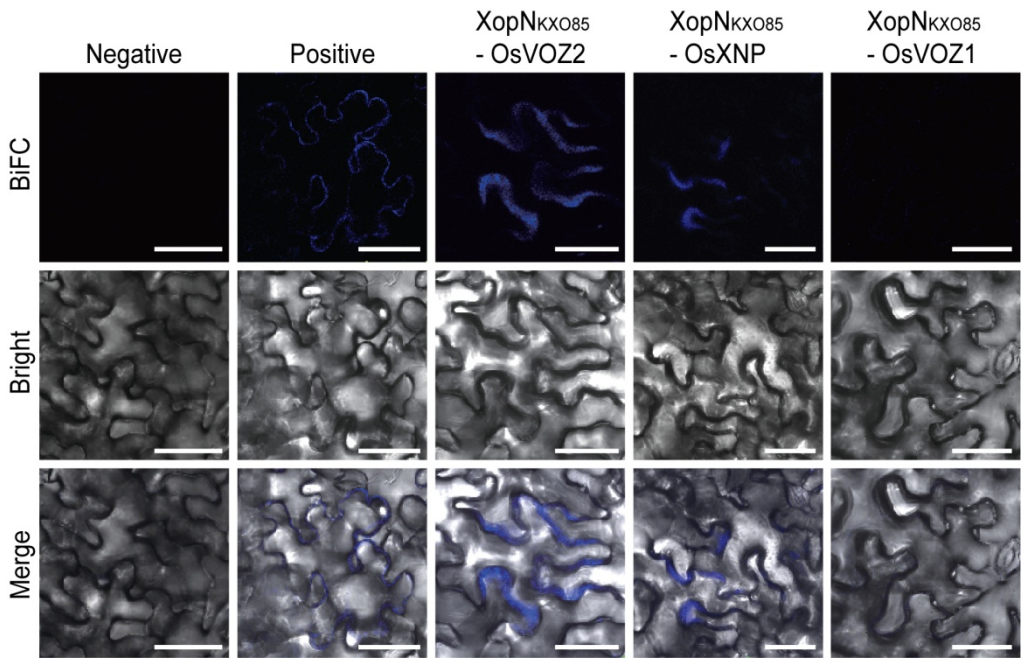
VI. Visualization of the interactions of OsVOZ2 and OsXNP with $XopN_{KXO85}$

A bimolecular fluorescence complementation (BiFC) assay was performed to examine the interactions between $XopN_{KXO85}$ and OsVOZ2 and $XopN_{KXO85}$ and OsXNP *in planta*. The coding sequences of *xopN*$_{KXO85}$, *OsVOZ2*, *OsXNP*, and *OsVOZ1* were cloned into pDEST-SCYNE(R)^{GW} and pDEST-SCYCE(R)^{GW} using the Gateway recombination system to yield pSCYNE(R)-XopN, pSCYCE(R)-OsVOZ2, pSCYCE(R)-OsXNP, and pSCYCE(R)-OsVOZ1, respectively (Table 1). When *Agrobacterium* cells carrying both plasmids were infiltrated into *N. benthamiana* leaves, the super cyan fluorescent protein (SCFP3A) signal was detected in the cytoplasm of the *N. benthamiana* cells (Fig. 9). As a positive control, I used the Cnx6 and Cnx7 interaction model to form a complex of molybdopterin synthase in *A. thaliana* using pEXP-SCYNE(R)-Cnx7 and pEXP-SCYCE(R)-Cnx6 (Gehl *et al*., 2009). These results indicate that $XopN_{KXO85}$ interacts with OsVOZ2 and OsXNP in the cytoplasm of *N. benthamiana* cells. However, $XopN_{KXO85}$ does not interact with OsVOZ1 in *N. benthamiana* cells (Fig. 9)

VII. Subcellular localization of $XopN_{KXO85}$, OsVOZ2, and OsXNP

To determine their subcellular localizations, $XopN_{KXO85}$ and OsVOZ2 were tagged with sGFP at their C-termini in pGWB5-XopN and pGWB5-OsVOZ2,

Fig. 9. BiFC analysis of $XopN_{KXO85}$ -OsVOZ2, $XopN_{KXO85}$ -OsXNP, and $XopN_{KXO85}$ -OsVOZ1 interactions in *N. benthamiana* leaves. Negative, pDEST-SCYNE(R)^{GW} + pDEST-SCYCE(R)^{GW}; positive, pEXP-SCYNE(R)-Cnx7 + pEXP-SCYCE(R)-Cnx6. Bars = 50 μm.


Negative
Positive
XopNKXO85 - OsVOZ2
XopNKXO85 - OsXNP
XopNKXO85 - OsVOZ1
BiFC
Bright
Merge

respectively (Table 1). In transient expression assays using *N. benthamiana* cells, sGFP signals from XopN-sGFP and OsVOZ2-sGFP were mostly detected in the cytoplasm. These data indicate that $XopN_{KXO85}$ and OsVOZ2 are localized in the cytoplasm. When subcellular localization of OsXNP was investigated with the sGFP-tagged OsXNP fusion in *N. benthamiana* leaves, OsXNP-sGFP signals were also detected in the cytoplasm (Fig. 10).

VIII. Interactions between $XopN_{KXO85}$ and OsVOZ2 are important for *Xoo* virulence in rice

To determine whether OsVOZ2 and its interactions with $XopN_{KXO85}$ are critical for *Xoo* virulence, the *OsVOZ2* knockout mutant line PFG_3A-07565 from the rice T-DNA Insertion Sequence Database (http://signal.salk.edu/cgi-bin/RiceGE) (Jeong *et al*., 2006) was inoculated with wild-type KXO85. In the mutant line PFG_3A-07565, T-DNA is inserted 929 nucleotides downstream from the translational start site of *OsVOZ2*. RT-PCR analysis detected *OsVOZ2* transcript in wild-type Dongjin but not in the *OsVOZ2* mutant line PFG_3A-07565 (Fig. 11), which confirmed knockout mutation in *OsVOZ2*. Wild-type KXO85 and $xopN_{KXO85}$ mutant strains were inoculated into wild-type Dongjin and the *OsVOZ2* mutant line, and the $xopN_{KXO85}$ mutant was shown to exhibit reduced virulence in the wild-type Dongjin. However, both strains

Fig. 10. Localization of $XopN_{KXO85}$, OsVOZ2, and OsXNP in plant cells. Subcellular localization of $XopN_{KXO85}$-sGFP, OsVOZ2-sGFP and OsXNP-sGFP fusion proteins in OsXNP in tobacco plant cells. Bars = 25 μm.

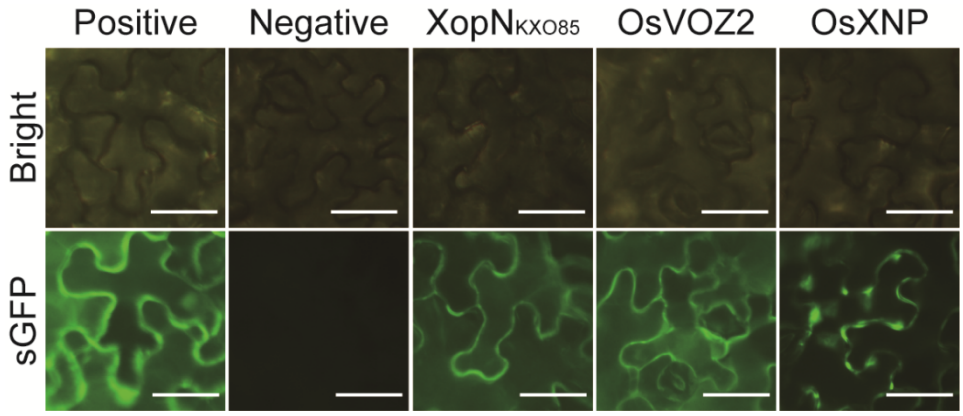
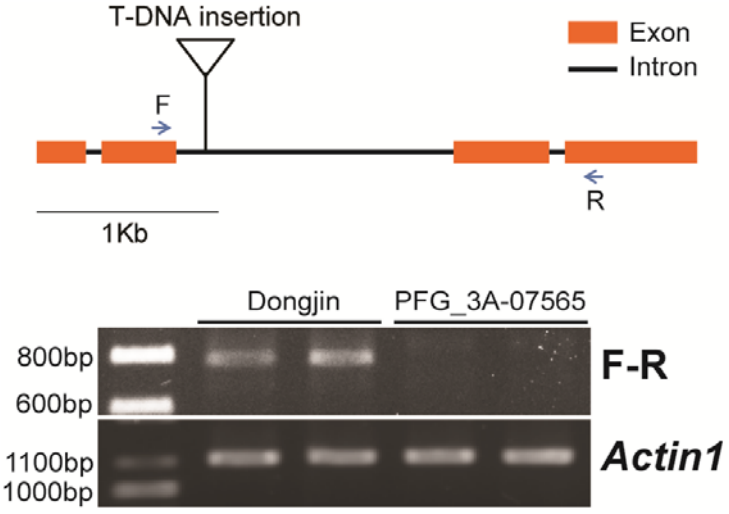
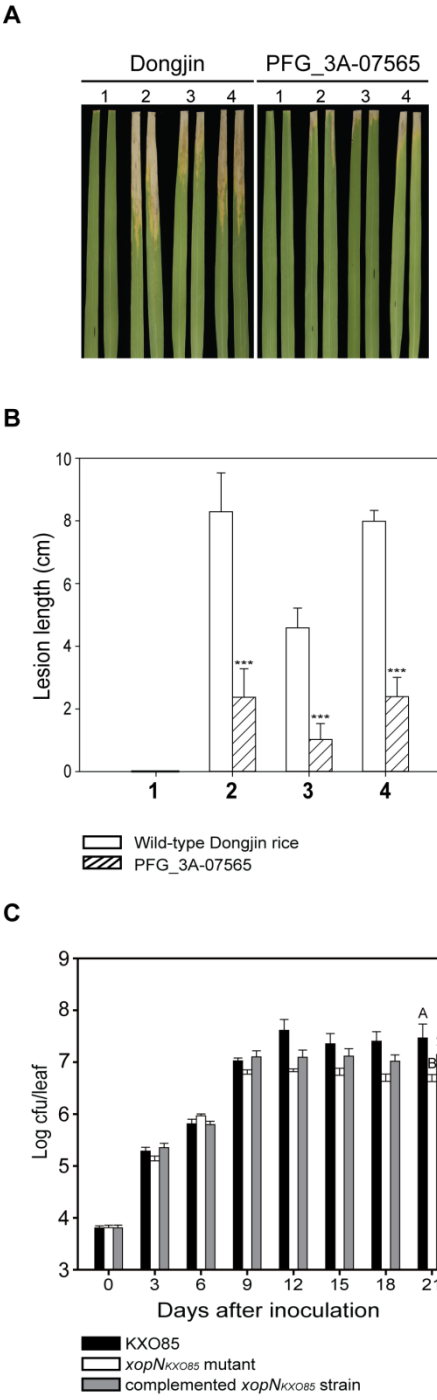

Positive
Negative
XopNKXO85
OsVOZ2
OsXNP
Bright
sGFP

Fig. 11. Schematic representation of T-DNA insertion in OsVOZ2 T_7 transgenic rice. *OsVOZ2* consists of four exons (orange boxes) and three introns (line between orange boxes). T-DNA is located in the second intron from the translational start site. F and R are primers used for RT-PCR analysis. RT-PCR analysis showed the expected size of *OsVOZ2* in the wild-type rice Dongjin but not in the *OsVOZ2* mutant rice PFG_3A-07565. Actin1 was used for normalization of cDNA quantity.


T-DNA insertion
Exon
Intron
F
R
1Kb
Dongjin
PFG_3A-07565
800bp
600bp
F-R
1100bp
1000bp
Actin1

showed significantly reduced disease severity in the *OsVOZ2* mutant line compared to the wild-type Dongjin (Fig. 12A and Fig. 12B). The $xopN_{KXO85}$ mutant was less virulent in the *OsVOZ2* mutant line than the wild-type KXO85 (Fig. 12A and Fig. 12B). The population of $xopN_{KXO85}$ mutant was smaller than that of wild-type KXO85 in the *OsVOZ2* mutant line (Fig. 12C). These results indicate that $XopN_{KXO85}$ is a virulence factor and that its interactions with OsVOZ2 are critical for *Xoo* virulence in rice.

Fig. 12. Virulence assay in the wild-type rice Dongjin and the OsVOZ2 mutant line PFG_3A-07565. (A) Virulence assay of the *xopN*$_{KXO85}$ mutant in wild-type rice and OsVOZ2 mutant rice. 1, Water; 2, KXO85; 3, KXO85 *xopN*$_{KXO85}$::EZ-Tn*5*; 4, KXO85 *xopN*$_{KXO85}$::EZ-Tn*5* (pML122G2). Photographs were taken 21 days after inoculation. (B) Measurement of disease severity in flag leaves of the wild-type rice Dongjin (□) and the OsVOZ2 mutant rice (▨). 1, Water; 2, KXO85; 3, KXO85 *xopN*$_{KXO85}$::EZ-Tn*5*; 4, KXO85 *xopN*$_{KXO85}$::EZ-Tn*5* (pML122G2). Lesion lengths were determined 21 days after inoculation. Vertical error bars indicate standard deviation (SD). Statistical significance was determined using two-way ANOVA as compared to wild-type Dongjin rice with Tukey HSD post test (***, $p<0.001$). (C) Growth patterns of KXO85, *xopN*$_{KXO85}$ mutant, and complemented *xopN*$_{KXO85}$ mutant in the flag leaves of OsVOZ2 mutant rice. The data are average values with three replicates, and vertical bars indicate the error ranges. Different letters at 21 day after inoculation indicate statically significant differences ($p<0.05$) as determined by one-way ANOVA ($P<0.001$) followed by a post-hoc Tukey HSD analysis.


A
Dongjin
PFG_3A-07565
1 2 3 4 1 2 3 4
B
Lesion length (cm)
10
8
6
4
2
0
***
***
***
1 2 3 4
Wild-type Dongjin rice
PFG_3A-07565
C
Log cfu/leaf
9
8
7
6
5
4
3
A
A
B
0 3 6 9 12 15 18 21
Days after inoculation
KXO85
xopNKXO85 mutant
complemented xopNKXO85 strain

DISCUSSION

There has been some confusion regarding the roles of Xop of *Xoo* because previous studies have used different *Xoo* strains. The *Xoo* PXO99^{A} strain has 18 non-TAL bacterial effectors (Song and Yang, 2010). Among these, $XopZ_{PXO99}$ acts as a virulence factor in the *Xoo* PXO99^{A} strain and suppresses plant basal defense mechanisms (Song and Yang, 2010). $XopR_{MAFF311018}$ was reported as a virulence factor in rice and inhibits the plant basal defense in *A. thaliana* (Akimoto-Tomiyama *et al*., 2012). Nine non-TAL effectors have been identified in the Chinese strain 13751, among which $XopR_{13751}$ has been shown to affect virulence in *Xoo* (Zhao *et al*., 2013). In the present study, I chose $XopN_{KXO85}$ to evaluate functional roles in the KXO85 strain and confirmed that it is secreted in an Hrp T3SS-dependent manner, translocated into the plant cytoplasm, and that its gene expression is regulated by $HrpX_{KXO85}$, as reported previously for other *Xoo* strains (Furutani *et al*., 2009). Among the Xop homologs in KXO85, I found that $XopN_{KXO85}$ is the most critical for *Xoo* virulence in the Korean strain KXO85. This result is similar to other reports indicating that $xopN_{Xcv}$ and $xopN_{Xcc}$ mutants show reduced virulence (Jiang *et al*., 2008; Kim *et al*., 2009).

It is worth noting that different Xop effectors from different *Xoo* strains

have been reported to be major Xops involved in *Xoo* virulence. Differences in genetic backgrounds of *Xoo* strains and rice cultivars used for virulence assays may explain why different research groups have reported different Xops as major virulence factors. For example, in one study, a mutation in the *xopN* homolog in *Xoo* PXO99^{A} did not alter disease severity in rice cultivar IR24 that was grown in a growth chamber for 4 weeks (Song and Yang, 2010). However, it should also be noted that differences in environmental conditions and various rice leaf stages used for inoculation of different *Xoo* strains may also result in different outcomes in virulence assays. In previous studies on *Xoo* PXO99^{A} and Chinese strain 13751, relatively young rice leaves were used for virulence assays in a growth chamber or a greenhouse (Song and Yang, 2010; Zhao *et al*., 2013), whereas I used flag leaves grown in a paddy field during the regular rice growing season. Disease severity induced by *Xoo* depends on rice leaf stage (Goel and Gupta, 1990; Mew, 1987; Sun *et al*., 2004; Zhang and Mew, 1985). In fact, I did not see marked differences in disease severity of $xopP1_{KXO85}$, $xopP2_{KXO85}$ and $xopN_{KXO85}$ mutants in young rice leaves, while there were significant differences in flag leaves. These observations correspond well with previous reports that the response to *Xoo* in rice depends on the age of the host (Goel and Gupta, 1990). Environmental conditions for growing rice and virulence assays are additional factors that may affect disease severity. It will be of interest to determine whether the $xopN_{PXO99}$ mutant of *Xoo* PXO99^{A}

shows differences in virulence assays when the mutant is inoculated into rice flag leaves.

Identification of target proteins of bacterial effectors in their hosts provides a basis for understanding effector functions and their roles in pathogenesis and host defense. $XopN_{Xcv}$ targets a tomato atypical receptor-like kinase1 (TARK1) and four tomato 14-3-3 isoforms (TFT1, TFT3, TFT5, and TFT6) to affect the defense signal mechanism (Kim *et al*., 2009). In *Xoo*, the Xoo1488 of *Xoo* MAFF311018 inhibits OsRLCK185 phosphorylation and the downstream MAPK signaling (Yamaguchi *et al*., 2013). Therefore, I postulated that $XopN_{KXO85}$ may interact with known kinases that are involved in signal transduction pathways in rice. However, unlike OsRLCK185 in rice, I found no kinase homologs as $XopN_{KXO85}$ targets but rather two previously unknown rice proteins, OsVOZ2 and OsXNP, were identified based on yeast two-hybrid analysis, pull-down, and BiFC assays.

The AtVOZs were first identified as novel transcription factors in *A. thaliana* (Mitsuda *et al*., 2004). AtVOZs interact with phytochrome B and accelerate flowering time in *A. thaliana* (Yasui *et al*., 2012). In the nuclei of *A. thaliana* cells, AtVOZ2 is controlled by light quality in a phytochrome-dependent manner (Yasui *et al*., 2012). In addition, AtVOZs are involved in controlling many stress reactions and changing the expression of various stress-related genes, such as

those related to drought or freezing responses and pathogens (Nakai *et al*., 2013). The genome of the wild-type rice Dongjin has an OsVOZ2 homolog, OsVOZ1, which is also an ortholog of AtVOZ2 and has conserved zinc finger amino acid residues (Mitsuda *et al*., 2004). OsVOZ1 and OsVOZ2 share 60.4% identity (Fig. 7). Due to the high degrees of similarity between OsVOZ1 and OsVOZ2, I performed BiFC analysis to determine whether OsVOZ1 is a target protein of $XopN_{KXO85}$. However, there was no evidence of an interaction between $XopN_{KXO85}$ and OsVOZ1 (Fig. 9).

AtVOZ2 interacts with five proteins in *A*. *thaliana*: phytochrome B (PHY B, At2g18790), guanine nucleotide-binding protein alpha-1 subunit (GP ALPHA1, At2g26300), guanine nucleotide-binding protein subunit beta (AGB1, At4g34460), pirin (PRN, At3g59220), and a hypothetical protein (At4g26410) (Yasui *et al*., 2012; Klopffleisch *et al*., 2011). The most apparent AtVOZ2-dependent phenotype is regulation of flowering period in *A*. *thaliana* after it interacts with phytochrome B (Yasui *et al*., 2012). However, it appears that OsVOZ2 is not involved in determining rice flowering time because I found no noticeable differences in flowering time between wild-type Dongjin and the *OsVOZ2* mutant rice line. Other than our findings indicating that OsVOZ2 is a target of $XopN_{KXO85}$ and is involved in *Xoo* virulence, no other functions have yet been reported in rice.

Another target of $XopN_{KXO85}$ is a putative thiamine synthase, OsXNP,

which is present as a single-copy gene in rice. The thiamine synthase gene is related to pathogen-induced defense-responsive protein 8 in indica rice cultivars. Treatment with thiamine induces callose deposition and hydrogen peroxide accumulation and triggers systemic acquired resistance and transient expression of pathogenesis-related genes against pathogen invasion in rice and several other plants (Ahn *et al*., 2005; Ahn *et al*., 2007). These phenomena are consistent with the observation that thiamine plays important roles in host defense mechanisms against pathogen infection. Therefore, I propose that $XopN_{KXO85}$ interacts with a putative thiamine synthase to hinder thiamine biosynthesis, thereby decreasing the defense of rice against *Xoo* infection. The target proteins of $XopN_{KXO85}$ in rice are completely different from the previously reported targets of $XopN_{Xcv}$ and $XopN_{Xcc}$. These observations indicate that XopN plays a common role as a virulence factor in *Xcv*, *Xcc*, and *Xoo* but functions in different ways in monocots and dicots, reflecting the different pathogen response mechanisms that arose during the coevolution of pathogens and their hosts.

In addition to roles of $XopN_{KXO85}$ as a virulence factor, OsVOZ2 is also important for *Xoo* virulence because wild-type KXO85 failed to successfully infect OsVOZ2 mutant rice. This suggests that interactions between $XopN_{KXO85}$ and OsVOZ2 in rice increases susceptibility to *Xoo* infection. That is, *Xoo* produces $XopN_{KXO85}$ as an effector molecule and utilizes the host protein OsVOZ2 for

successful infection and increased virulence. Although the functions of OsVOZ2 are not fully understood in the interactions between *Xoo* and rice, it is evident that OsVOZ2 is a key factor in *Xoo* virulence in rice.

LITERATURE CITED

Ahn I-P, Kim S, Lee Y-H (2005) Vitamin B1 functions as an activator of plant disease resistance. *Plant Physiol* 138: 1505-1515.

Ahn I-P, Kim S, Lee Y-H, Suh S-C (2007) Vitamin B1-induced priming is dependent on hydrogen peroxide and the NPR1 gene in Arabidopsis. *Plant Physiol* 143: 838-848.

Akimoto-Tomiyama C, Furutani A, Tsuge S, Washington E J, Nishizawa Y, et al. (2012) XopR, a type III effector secreted by *Xanthomonas oryzae* pv. *oryzae*, suppresses microbe-associated molecular pattern-triggered immunity in *Arabidopsis thaliana*. Mol Plant Microbe Interact 25: 505-514.

Alfano JR, Collmer A (2004) Type III secretion system effector proteins: Double agents in bacterial disease and plant defense. *Annu Rev Phytopathol* 42: 385-414.

Bai J, Choi SH, Ponciano G, Leung H, Leach JE (2000) *Xanthomonas oryzae* pv. *oryzae* avirulence genes contribute differently and specifically to pathogen aggressiveness. *Mol Plant Microbe Interact* 13: 1322-1329.

Boch J, Bonas U (2010) *Xanthomonas* AvrBs3 family-type III effectors: discovery and unction. *Annu Rev Phytopathol* 48: 419-436.

Bogdanove AJ, Schornack S, Lahaye T (2010) TAL effectors: finding plant genes for disease and defense. *Curr Opin Plant Biol* 13: 394-401.

Boureau T, Siamer S, Perino C, Gaubert S, Patrit O, et al. (2011) The HrpN effector of *Erwinia amylovora*, which is involved in type III translocation, contributes directly or indirectly to callose elicitation on apple leaves. *Mol Plant Microbe Interact* 24: 577-584.

Büttner D, Bonas U (2002) Port of entry-the type III secretion translocon. *Trends Microbiol* 10: 186-192.

Büttner D, He SY (2009) Type III protein secretion in plant pathogenic bacteria. *Plant Physiol* 150: 1656-1664.

Canonne J, Marino D, Jauneau A, Pouzet C, Briere C, et al. (2011) The *Xanthomonas* type III effector XopD targets the *Arabidopsis* transcription factor MYB30 to suppress plant defense. *Plant Cell* 23: 3498-3511.

Cho JI, Ryoo N, Eom JS, Lee DW, Kim HB, et al. (2009) Role of the rice

hexokinases OsHXK5 and OsHXK6 as glucose sensors. *Plant Physiol* 149: 745-759.

Crabill E, Karpisek A, Alfano JR (2012) The *Pseudomonas syringae* HrpJ protein controls the secretion of type III translocator proteins and has a virulence role inside plant cells. *Mol Microbiol* 85: 225-238.

Furutani A, Takaoka M, Sanada H, Noguchi Y, Oku T, et al. (2009) Identification of novel type III secretion effectors in *Xanthomonas oryzae* pv. *oryzae*. *Mol Plant Microbe Interact* 22: 96-106.

Gala´n JE, Collmer A (1999) Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* 284: 1322-1328.

Gehl C, Waadt R, Kudla J, Mendel R -R, Hänsch R (2009) New gateway vectors f or high throughput analyses of protein-protein interactions by bimolecular f luorescence complementation. *Mol Plant* 2: 1051-1058.

Ghosh P (2004) Process of protein transport by the type III secretion system. *Microbiol Mol Biol Rev* 68: 771-795.

Goel RK, Gupta AK (1990) Host age in relation to resistance in rice to bacterial

blight caused by *Xanthomonas campestris* pv. *oryzae*. *Trop Agric* 67: 368-370.

Göhre V, Robatzek S (2008) Breaking the barriers: Microbial effector molecules subvert plant immunity. *Annu Rev Phytopathol* 46: 189-215.

Gu K, Yang B, Tian D, Wu L, Wang D, et al. (2005) R gene expression induced by a type-III effector triggers disease resistance in rice. *Nature* 435: 1122-1125.

Hossain MA, Cho JI, Han M, Ahn CH, Jeon JS, et al. (2010) The ABRE-binding bZIP transcription factor OsABF2 is a positive regulator of abiotic stress and ABA signaling in rice. *J Plant Physiol* 167: 1512-1520.

Hwang IS, Kim NH, Choi DS, Hwang BK (2012) Overexpression of *Xanthomonas campestris* pv. *vesicatoria* effector AvrBsT in Arabidopsis triggers plant cell death, disease and defense responses. *Planta* 236: 1191-1204.

Hwang I, Sheen J (2001) Two-component circuitry in Arabidopsis cytokinin signal transduction. *Nature* 413: 383-389.

Jeong DH, An S, Park S, Kang HG, Park GG, et al. (2006) Generation of a flanking

sequence-tag database for activation tagging lines in japonica rice. *Plant J* 45: 123-132.

Jiang B-L, He Y-Q, Cen W-J, Wei H-Y, Jiang G-F, et al. (2008) The type III secretion effector XopXccN of *Xanthomonas campestris* pv. *campestris* is required for full virulence. *Res Microbiol* 159: 216-220.

Karimi M, Inze D, Depicker A (2002) Gateway vectors for *Agrobacterium*-mediated plant transformation. *Trends Plant Sci* 7: 193-195.

Kauffman HE, Reddy APK, Hsieh SPY, Merca SD (1973) An improved technique for evaluating resistance of rice varieties to *Xanthomonas oryzae*. *Plant Dis Rep* 57: 537-541.

Kim J-G, Li X, Roden JA, Taylor KW, Aakre CD, et al. (2009) *Xanthomonas* T3S effector XopN suppresses PAMP-triggered immunity and interacts with a tomato atypical receptor-like kinase and TFT1. *Plant Cell* 21: 1305-1323.

Kim J-G, Stork W, Mudgett MB (2013) *Xanthomonas* type III effector XopD desumoylates tomato transcription factor SIERF4 to suppress ethylene responses and promote pathogen growth. Cell Host Microbe 13: 143-154.

Kim J-G, Taylor KW, Hotson A, Keegan M, Schmelz EA, et al. (2008) XopD SUMO protease affects host transcription, promotes pathogen growth, and delays symptom development in *Xanthomonas*-infected tomato leaves. *Plant Cell* 20: 1915-1929.

Klopffleisch K, Phan N, Augustin K, Bayne RS, Booker KS, et al. (2011) Arabidopsis G-protein interactome reveals connections to cell wall carbohydrates and morphogenesis. *Mol Syst Biol* 7: 532.

Labes M, Pühler A, Simon R (1990) A new family of RSF1010-derived expression and *lac*-fusion broad-host-range vectors for Gram-negative bacteria. *Gene* 89: 37-46.

Lee B-M, Park Y-J, Park D-S, Kang H-W, Kim J-G, et al. (2005) The genome sequence of *Xanthomonas oryzae* pathovar *oryzae* KACC10331, the bacterial blight pathogen of rice. *Nucleic Acids Res* 33: 577-586.

Metz M, Dahlbeck D, Morales CQ, Sady BA, Clark ET, et al. (2005) The conserved *Xanthomonas campestris* pv. *vesicatoria* effector protein XopX is a virulence factor and suppresses host defense in *Nicotiana benthamiana*. *Plant J* 41: 801-814.

Mew TW (1987) Current status and future prospects of research on bacterial blight of rice. *Annu Rev Phytopathol* 25: 359-382.

Meyer D, Cunnac S, Gueneron M, Declercq C, Van Gijsegem F, et al. (2006) PopF1 and PopF2, two proteins secreted by the type III protein secretion system of *Ralstonia solanacearum*, are translocators belonging to the HrpF/NopX family. *J Bacteriol* 188: 4903-4917.

Mitsuda N, Hisabori T, Takeyasu K, Sato MH (2004) VOZ; Isolation and characterization of novel vascular plant transcription factors with a one-zinc finger from *Arabidopsis thaliana*. *Plant Cell Physiol* 45: 845-854.

Mudgett MB (2005) New insights to the function of phytopathogenic bacterial type III effectors in plants. *Annu Rev Plant Biol* 56: 509-531.

Mudgett MB, Chesnokova O, Dahlbeck D, Clark ET, Rossier O, et al. (2000) Molecular signals required for type III secretion and translocation of the *Xanthomonas campestris* AvrBs2 protein to pepper plants. *Proc Natl Acad Sci USA* 97: 13324-13329.

Nakagawa T, Kurose T, Hino T, Tanaka K, Kawamukai M, et al. (2007) Development of series of gateway binary vectors, pGWBs, for realizing

efficient construction of fusion genes for plant transformation. *J Biosci Bioeng* 104: 34-41.

Nakai Y, Nakahira Y, Sumida H, Takebayashi K, Nagasawa Y, et al. (2013) Vascular plant one-zinc-finger protein 1/2 transcription factors regulate abiotic and biotic stress responses in Arabidopsis. *Plant J* 73: 761-775.

Petnicki-Ocwieja T, van Dijk K, Alfano JR (2005) The *hrpK* operon of *Pseudomonas syringae* pv. *tomato* DC3000 encodes two proteins secreted by the type III (Hrp) protein secretion system: HopB1 and HrpK, a putative type III translocator. *J Bacteriol* 187: 649-663.

Roden JA, Belt B, Ross JB, Tachibana T, Vargas J, et al. (2004) A genetic screen to isolate type III effectors translocated into pepper cells during *Xanthomonas* infection. *Proc Natl Acad Sci USA* 101: 16624-16629.

Roden JA, Eardley L, Hotson A, Cao Y, Mudgett MB (2004) Characterization of the *Xanthomonas* AvrXv4 effector, a SUMO protease translocated into plant cells. *Mol Plant Microbe Interact* 17: 633-643.

Romer P, Recht S, Strauss T, Elsaesser J, Schornack S, et al. (2010) Promoter elements of rice susceptibility genes are bound and activated by specific

TAL effectors from the bacterial blight pathogen, *Xanthomonas oryzae* pv. *oryzae*. *New Phytol* 187: 1048-1057.

Sambrook J, Fritsch EF, Maniatis T (1997) Molecular cloning: A laboratory mannual. *Cold Spring Harbor Laboratory Press*, Cold Spring Harbor, NY, USA

Scholze H, Boch J (2011) TAL effectors are remote controls for gene activation. *Curr Opin Microbiol* 14:47-53.

Seo Y-S, Chern M, Bartley LE, Han M, Jung K-H, et al. (2011) Towards establishment of a rice stress response interactome. *PLoS Genet* 7(4): e1002020. doi:10.1371/journal.pgen.1002020.

Song C, Yang B (2010) Mutagenesis of 18 TypeIII effectors reveals virulence function of $XopZ_{PXO99}$ in *Xanthomonas oryzae* pv. *oryzae*. *Mol Plant Microbe Interact* 23: 893-902.

Sugio A, Yang B, White FF (2005) Characterization of the *hrpF* pathogenicity peninsula of *Xanthomonas oryzae* pv. *oryzae*. *Mol Plant Microbe Interact* 18: 546-554.

Sun X, Cao Y, Yang Z, Xu C, Li X, et al. (2004) *Xa26*, a gene conferring resistance to *Xanthomonas oryzae* pv. *oryzae* in rice, encodes an LRR receptor kinase-like protein. *Plant J* 37: 517-527.

Truernit E and Haseloff J (2008) A simple way to identify non-viable cells within living plant tissue using confocal microscopy. *Plant Methods* 4: 15.

Tsuge S, Furutani A., Fukunaka R, Oku T, Tsuno K, et al. (2002) Expression of *Xanthomonas oryzae* pv. *oryzae hrp* genes in XOM2, a novel synthetic medium. *J Gen Plant Pathol* 68: 363-371.

Verdier V, Triplett LR, Hummel AW, Corral R, Cernadas RA, et al. (2012) Transcription activator-like (TAL) effectors targeting OsSWEET genes enhance virulence on diverse rice (*Oryza sativa*) varieties when expressed individually in a TAL effector-deficient strain of *Xanthomonas oryzae*. *New Phytol* 196: 1197-1207.

White FF, Potnis N, Jones JB, Koebnik R (2009) The type III effectors of *Xanthomonas*. *Mol Plant Pathol* 10: 749-766.

Yamaguchi K, Yamada K, Ishikawa K, Yoshimura S, Hayashi N, et al. (2013) A receptor-like cytoplasmic kinase targeted by a plant pathogen effector is

directly phosphorylated by the chitin receptor and mediates rice imunity. Cell Host Microbe 13: 347-357.

Yasui Y, Mukougawa K, Uemoto M, Yokofuji A, Suzuri R, et al. (2012) The phytochrome-interacting VASCULAR PLANT ONE-ZINC FINGER1 and VOZ2 redundantly regulate flowering in Arabidopsis. *Plant cell* 24: 3248-3263.

Yu Y, Streubel J, Balzergue S, Champion A, Boch J, Koebnik R, et al. (2011) Colonization of rice leaf blades by an African strain of *Xanthomonas oryzae* pv. *oryzae* depends on a new a TAL effector that induces the rice nodulin-3 Os11N3 gene. *Mol Plant Microbe Interact* 24: 1102-1113.

Zhang Q, Mew TW (1985) Adult-plant resistance of rice cultivars to bacterial blight. *Plant Dis* 69: 896-898.

Zhao S, Mo WL, Wu F, Tang W, Tang JL, et al. (2013) Identification of non-TAL effectors in *Xanthomonas oryzae* pv. *oryzae* Chinese strain 13751 and analysis of their role in the bacterial virulence. *World J Microbiol Biotechnol* 29: 733-744.

Zhu W, Magbanua MM, White FF (2000) Identification of Two Novel

hrp-Associated Genes in the *hrp* Gene Cluster of *Xanthomonas oryzae* pv. *oryzae*. *J Bacteriol* 182: 1844-1853.

벼흰잎마름병균이 분비하는 Type III Effector XopN과 벼의 단백질 OsVOZ2, Putative Thiamine Synthase의 상호작용

정 훈

초 록

쌀은 세계 5대 주식(主食) 중의 하나로 인류발전과 함께 기나긴 역사를 가지고 있는 식량 작물이다. 벼는 많은 아시아 국가 및 북미, 그리고 아프리카에 이르기까지 재배 면적이 넓으며, 또한 벼에 병을 일으키는 병원균도 넓게 분포하고 있다. 벼에 병을 일으키는 대표적인 세균병인 벼흰잎마름병 (Bacterial leaf blight)은 병원세균 *Xanthomonas oryzae* pv. *oryzae* (*Xoo*)에 의해 벼의 잎이 말라 죽는 도관병으로 잎의 수공이나 기공, 그리고 상처를 통해 병원균이 침입을 하고, 벼에 감염이 심할 시 쌀의 수확량의 감소에 크나큰 영향을 미친다. 이와 같은 심각한 피해로 인하여 많은 연구자들이 벼흰잎마름병을

억제하려는 연구와 노력을 해 왔고 근래에 이르러서는 이 병원균이 가지는 유전자 서열을 전부 해독하여 이 후 이 세균이 가지는 유전자의 기능 연구에 도움을 주게 되었다.

본 연구는 *Xoo* KXO85 의 유전자 정보를 바탕으로 이 세균이 가진 단백질 제 3형 분비체계 (Type III secretion system)를 이용하여 기주인 벼의 세포내로 들어가 기주의 저항성 또는 감수성에 영향을 미치는 신호전달에 관여하는 단백질인 Bacterial effector protein들 중 Non-TAL effetor인 *Xanthomonas* outer protein(Xop)들을 선별하고 단백질의 기능이 훼손되었을 때 병원성에 어떤 영향을 미치는지를 확인하였다. 또한 이러한 Xop effector들 중 특별히 XopN은 벼의 세포내로 들어가 벼의 두 단백질과 상호 작용하여 병원성에 영향을 주는 것을 확인할 수 있었다.

Xoo KXO85에는 전체 유전자들 중에서 18개 정도의 Xop effector들을 만드는 것으로 추정되는 유전자들이 있으며 본 논문에서는 그 중 5개의 xop (*xopQ*, *xopX*, *xopP1*, *xopP2*, *xopN*)유전자들을 선별하여 병원성에 영향을 미치는 정도를 알아보기 위해 먼저 EZ-Tn5를 이용한 transposon mutagenesis를 시도하였다. 이렇게

만들어진 *xop* mutant들을 대상으로 약 3개월 정도 논포장에서 키운 벼의 잎에 접종하였을때 wild-type (WT) strain인 *Xoo* KXO85에 비해 병원성의 큰 차이가 없었다. 그러나 4개월 정도 키운 벼에서 이삭이 나오고 그 옆에 생긴 지엽에 *xop* mutant들을 접종했을 때 WT인 *Xoo* KXO85에 비해 *xopN, xopP2*, 그리고 *xopP1 mutant*들이 현저하게 병원성이 감소함을 알 수 있었고, 다시 완전한 *xop*유전자를 plasmid에 싣어 각 mutant에 넣어 주었을 때 병원성이 회복됨을 알 수 있었다. 이 결과를 통하여 XopN, XopP2, 그리고 XopP1 effector 단백질들은 벼의 특정한 시기의 잎에 의존적으로 병을 일으키는 데 관여함으로 사료된다. 특별히 *xopN* mutant에 대해서 접종 후 21일째까지 벼 안의 세균 밀도를 측정한 결과 WT인 *Xoo* KXO85에 비해 세균밀도가 떨어짐을 확인하였고, 이 후 모든 실험에서는 XopN effector 단백질에 초점을 맞추어 벼의 세포안으로 들어가 기주의 특정 단백질과 상호작용하는지 알아보기 위해 분자생물학적 기법들을 사용하여 연구를 수행하였다.

먼저 XopN 단백질이 Type III secretion system를 통하여 실제 기주인 벼의 세포내로 들어가는 지 확인하기 위해 Cya reporter system을 이용한 벼에 실험 결과에서 bacterial effector protein임이

증명되었고, 다른 *Xanthomonas* 속 세균들의 Xop effector들과 마찬가지로 HrpX에 의해 *xopN*의 발현이 조절됨을 RT-PCR과 qRT-PCR을 통해서 증명하였다. 위의 두 결과는 또한 일본 균주인 *Xoo* MAFF311018의 Xop effector들에서도 동일한 결과를 나타내는 것으로 보고되어 그 신뢰성을 더하여 준다. 이 후 XopN과 상호 작용하는 벼 단백질을 찾기 위하여 단백질과 단백질간의 결합을 알아보는 실험인 Yeast two-hybrid (Y2H) screening을 통하여 두 개의 동진벼 단백질을 찾을 수 있었다. 하나는, *Oryza sativa* vascular plant one zinc finger protein 2 (OsVOZ2) 이고 다른 하나는 thiamine synthase로 추정되는 단백질로 XopN과 결합한다고 하여 *Oryza sativa* $XopN_{KXO85}$ binding protein (OsXNP) 으로 명명하였다. Y2H를 통해 XopN과 결합한다고 판단되는 두 단백질이 식물체안에서 bacterial effector protein인 XopN과 상호 작용하는 것을 육안으로 관찰하기 위해 bimolecular fluorescence complementation (BiFC) assay를 수행하여 confocal laser scanning microscope로 확인 하였으며, 또한 *in vivo* pull-down asasy를 통하여 식물안에서 실제로 결합함을 증명하였다.

벼와 병원균인 *Xoo*의 상호 작용에 있어 OsVOZ2의 중요한 역할을 알아보기 위하여 OsVOZ2가 동진벼에서 Knock-out된 *OsVOZ2* mutant line PFG_3A-07565 벼를 이용하여 WT strain인 *Xoo* KXO85와 *xopN* mutant 를 접종하였을 때 *OsVOZ2* mutant line PFG_3A-07565 벼에서 현저하게 WT 동진벼에 비해 병원성과 세균의 밀도가 감소함을 알 수 있었다. 이러한 결과는 벼에서 XopN과 OsVOZ2가 각 각 독립적으로 또는 둘이 같이 *Xoo*의 병원성에 중요한 역할을 하는 것으로 판단 되는 바이다.

주요어 : 벼흰잎마름병균 (*Xanthomonas oryzae pv. oryzae*), *xop genes,* 단백질 제 3형 분비체계 (TypeIII secretion system), yeast two-hybrid, bimolecular fluorescence complementation, pull-down assay

학번: 2005-31098