

XerR, a negative regulator of XccR in *Xanthomonas campestris* pv. *campestris*, relieves its repressor function *in planta*

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We previously reported that XccR, a LuxR-type regulator of *Xanthomonas campestris* pv. *campestris* (*Xcc*), activates the downstream proline iminopeptidase virulence gene (*pip*) in response to certain host plant factor(s). In this report, we further show that the expression of the *xccR* gene was repressed in the culture medium by an NtrC-type response regulator, which we named XerR (XccR expression-repressor), and that this repression was relieved when the bacteria were grown *in planta*. Such a regulatory mechanism is reinforced by the observations that XerR directly bound to the *xccR* promoter *in vitro*, and that mutations at the phosphorylation-related residues of XerR resulted in the loss of its repressor function. Furthermore, the expression level of *xccR* increased even in XerR-overexpressing *Xcc* cells when they were vacuum infiltrated into cabbage plants. We also preliminarily characterized the host factor(s) involved in the above mentioned interactions between *Xcc* and the host plant, showing that a plant material(s) with molecular weight(s) less than 1 kDa abolished the binding of XerR to the *xccR* promoter, while the same material enhanced the binding of XccR to the *luxXc* box in the *pip* promoter. Taken together, our results implicate XerR in a new layer of the regulatory mechanism controlling the expression of the virulence-related *xccR/pip* locus and provide clues to the identification of plant signal molecules that interact with XerR and XccR to enhance the virulence of *Xcc*.

Keywords: *Xcc*; NtrC-like regulator; LuxR-like regulator; proline iminopeptidase; pathogen-host interaction; plant signal(s)
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Introduction

In the past few decades it has become obvious that bacteria can display sophisticated group behaviors and form communities in their natural niches in response to constant changes in physical, chemical and biological environments [1-3]. The regulation of gene expression mediated by signaling molecules and regulatory proteins

in a bacterial population density-dependent manner is referred to as quorum sensing (QS). The first QS system in Gram-negative bacteria was observed in *Vibrio fischeri*, which contains a LuxR regulator and a cognate LuxI synthase responsible for producing autoinducer signal molecules *N*-acylhomoserine lactones (AHLs) [4-6]. To date, QS-dependent functions have been studied in a wide variety of bacteria that control diverse bacterial processes, including virulence, sporulation, plasmid transfer, biosynthesis of antibiotics, as well as plant nodulation [7-9]. It is now increasingly evident that QS is a complicated group behavior of bacteria for producing, sensing and responding to multifarious chemical signals to increase their chances of survival and propagation [7, 8]. In other cases, QS-mediated communications are also involved in interactions between bacterial species and between bacteria and their hosts. For example, γ -amino butyric acid (GABA) produced by plant induces the expression of the *attKLM* operon in *Agrobacterium tumefaciens*,

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which causes the bacterium to destroy its own QS signal [10], while L-proline interferes with the import of GABA and antagonizes the degradation of bacterial QS signal, 3-oxo-octanoylhomoserine lactone [11].

A genomic survey of Proteobacteria showed that there are numerous bacteria that do not encode a cognate LuxI synthase for AHLs [12]. As a result, the unpaired LuxR-like proteins designated as LuxR-family orphans or 'solos' have been studied [13, 14]. LuxR solos such as ExpR of *Sinorhizobium meliloti*, BisR of *Rhizobium leguminosarum* pv. *viciae* and QscR of *Pseudomonas aeruginosa*, respond to AHL signals produced by the bacteria themselves [15-17]. In addition, SdiA in *Salmonella*, *Escherichia* and *Klebsiella* are able to bind and detect AHLs produced by other bacterial species [18]. Interestingly, accumulating evidence from recent studies supports the idea that, apart from playing important roles in sensing AHL-like autoinducers, LuxR-like solos could potentially sense non-AHL signaling molecules as well [13, 14, 19].

As a special LuxR-like solo, XccR of the plant pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*) is required for activating the expression of the downstream proline iminopeptidase gene (*pip*) through binding to the *luxXc* box in the *pip* promoter, and this activation is enhanced by plant host factors [20]. The *xccR/pip* locus is different from the classical *luxR/luxI* system in that *pip* is a virulence-related gene, rather than a gene for producing AHL signals. The *xccR/pip*-like locus has been found in several other bacteria, such as *S. meliloti*, *Rhodospirillum rubrum*, *R. leguminosarum* and *P. syringae* [20]. More particularly, the *oryR/pip* locus of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) behaves very much like the *xccR/pip* locus. In addition, the solubility of OryR is enhanced by a rice extract with molecular weights less than 1 kDa [21]. OryR also positively regulates the expression of a cell wall-degrading cellobiosidase gene for optimal pathogenicity [22].

In this study, we explored the bacterial upstream factor(s) and the host plant signals regulating the expression of the *xccR/pip* locus. By screening a genome-scale Tn5-insertion library of an *Xcc* strain harboring an *xccR* promoter-*gusA* fusion, we identified an NtrC-type transcriptional regulator XC_3760 (named XerR, XccR expression-related, repressor) as a repressor of the *xccR/pip* locus. NtrC-type proteins have been recognized as enhancer-binding proteins in phosphorylated forms; they are involved in nitrogen assimilation, biofilm formation, bioluminescence and QS regulatory system, and thus their functions are expected to be pleiotropic [23-26]. Furthermore, we showed that the repressor function of XerR was relieved in the presence of the host plant ex-

tract with molecular weights less than 1 kDa, and that the same plant extract enhanced the binding of XccR to the *pip* promoter sequence. Our results expand the regulatory machinery controlling the expression of the pathogenicity-related *xccR/pip* locus and provide new insights into how *Xcc* senses host signals to regulate its infectivity.

Results

Genetic screening of *xccR* expression reveals a repressor, XerR

To identify factors that regulate the expression of *xccR*, which directs the expression of the virulence gene *pip* in *Xcc*, we designed an antibiotic-coupled transposon screen. The chromosomal *xccR* promoter (*xccR*-P)/*gusA* fusion strain (*Xcc* 8177) was mutated with the EZ-Tn5 transposon that contains the dihydrofolate reductase (DHFR) gene for conferring trimethoprim resistance. From 20 000 transposon-insertion mutants, we selected one that pointed to a possible repressor of *xccR* expression. Analysis of the flanking sequences of the mutated gene indicated the gene was XC_3760 encoding a transcriptional regulator of the NtrC family, which we designated as *xerR* in this paper.

XerR is a putative 433 amino acid protein with a predicted molecular weight of 48.2 kDa and belongs to the two-component signal transduction system (TCSTS) response regulator (RR) NtrC family [27]. BLAST search against the databases revealed that XerR is highly conserved in all *Xanthomonas* species and shares significant sequence similarities with NtrC family proteins from *Bordetella bronchiseptica* (61%), *Ralstonia solanacearum* (60%) and *Burkholderia xenovorans* (58%; NCBI Blast: <http://www.ncbi.nlm.nih.gov/BLAST/>). Analyses with Pfam indicated that XerR contains an N-terminal receiver domain that has the conserved Asp site for phosphorylation, a central ATP-binding AAA+ domain that hydrolyzes ATP to generate energy, and a C-terminal domain containing a helix-turn-helix motif for DNA binding [23]. Multiple sequence alignments between XerR and CheY in *Escherichia coli* [28], NtrC in *Salmonella typhimurium* [29] and LuxO in *Vibrio harveyi* [25], revealed the highly conserved residues Asp-17, Asp-60 and Phe-106 in the receiver motif (Figure 1A). These residues were shown to be critical for the functioning of the phosphorylated protein, and Asp-60 was proposed to be the phosphorylated site. Although the histidine kinase and its cognate RR are usually linked in one operon [30], a search of the genome did not suggest that the *Xcc* chromosome encodes a cognate TCSTS sensor protein in close vicinity of the *xerR* sequence. It was the overall structure and location of *xerR* that prompted us to study

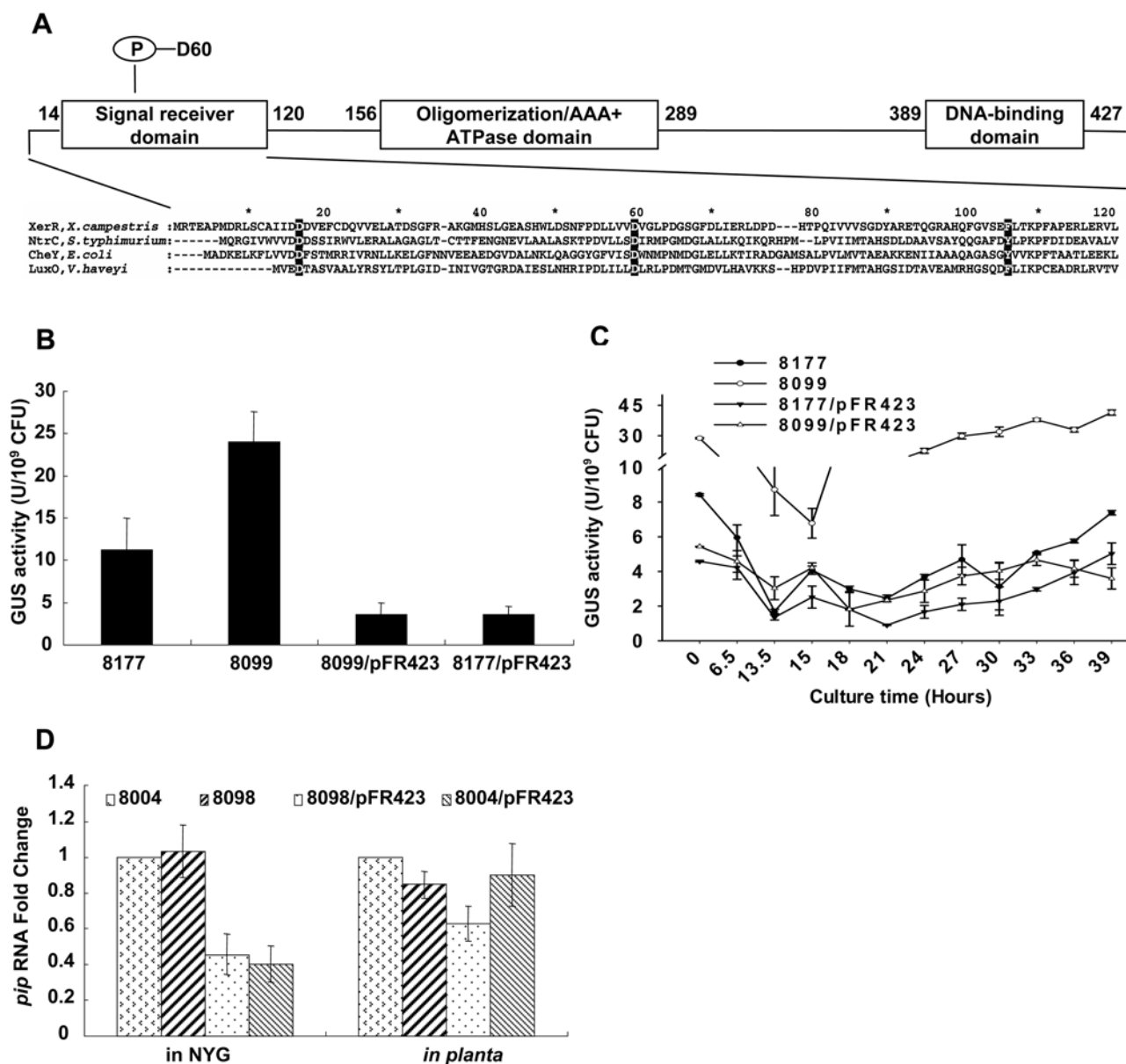


Figure 1 XerR is required for repression of *xccR* and *pip* transcription in medium. **(A)** The domain organization of XerR and the sequence of receiver domain. Three putative modular components of XerR are shown in the diagram. Multiple amino acid sequence alignments between XerR and NtrC in *S. typhimurium*, CheY in *E. coli* and LuxO in *V. harveyi* are shown at the bottom of the diagram. The residues altered by site-directed mutagenesis are shaded in black, and the putative phosphorylation site (Asp-60) is marked. **(B)** GUS expression levels in different Xcc strains were assayed by enzymatic activities. *xerR* in-frame deletion mutant Xcc 8099 increased the GUS activity compared to that of Xcc 8177. Xcc 8099 and Xcc 8177 carrying the *xerR* gene in pHM1 plasmid (Xcc 8099/pFR423 and Xcc 8177/pFR423) exhibited reduced GUS activities. All the strains were harvested at OD₆₀₀ of 2.0 in NYG medium. Relative GUS activity units were defined as nM 4-methylumbelliferyl/min/10⁹ cells. The means and standard deviations were calculated from the data derived from at least nine independent experiments. **(C)** Expression of *xccR*-P/*gusA* in Xcc 8099 was density dependent when grown in NYG medium. GUS activities of different strains were assayed at different time points. The mean and standard deviation were calculated from the data derived from three independent experiments. **(D)** Expression levels of *pip* in different Xcc strains in medium and *in planta*. Relative transcriptional levels of *pip* were quantified by real-time RT-PCR. In NYG medium, RNA were extracted from the cultured strains at a cell density of OD₆₀₀ = 1.5-2.0. *In planta*, RNA was isolated from vacuum-infiltrated cabbage leaves 30 h post infiltration. Measurements were normalized by the wild-type values and fold differences were plotted. Each sample was assayed in triplicate.

scriptional sites and ensures that the gene is turned off in an efficient and specific manner [32, 33].

Phosphorylation-related residues of XerR are essential for its repressor function

In vivo and *in vitro* experiments indicated that XerR can efficiently repress the expression of *xccR*. To characterize the repressor function-related motifs, we first tested whether the N-terminal part or the C-terminal part of XerR is critical in regulating transcription of *xccR*. We

constructed an *xerR* RR domain (N-terminal amino acids 14-120) deletion mutant (*xerR* Δ RR) and an HTH domain (C-terminal amino acids 385-433) deletion mutant (*xerR* Δ HTH), and assayed GUS activities under the control of the *xccR* promoter in these two *xerR* deletion strains. The results showed that the GUS activities produced by *xerR* Δ RR and *xerR* Δ HTH were 3.06 times and 2.97 times, respectively, of that of *Xcc* 8177 in NYG medium (Figure 3A), indicating that removal of the receiver or DNA-binding domain results in an inactive XerR protein and

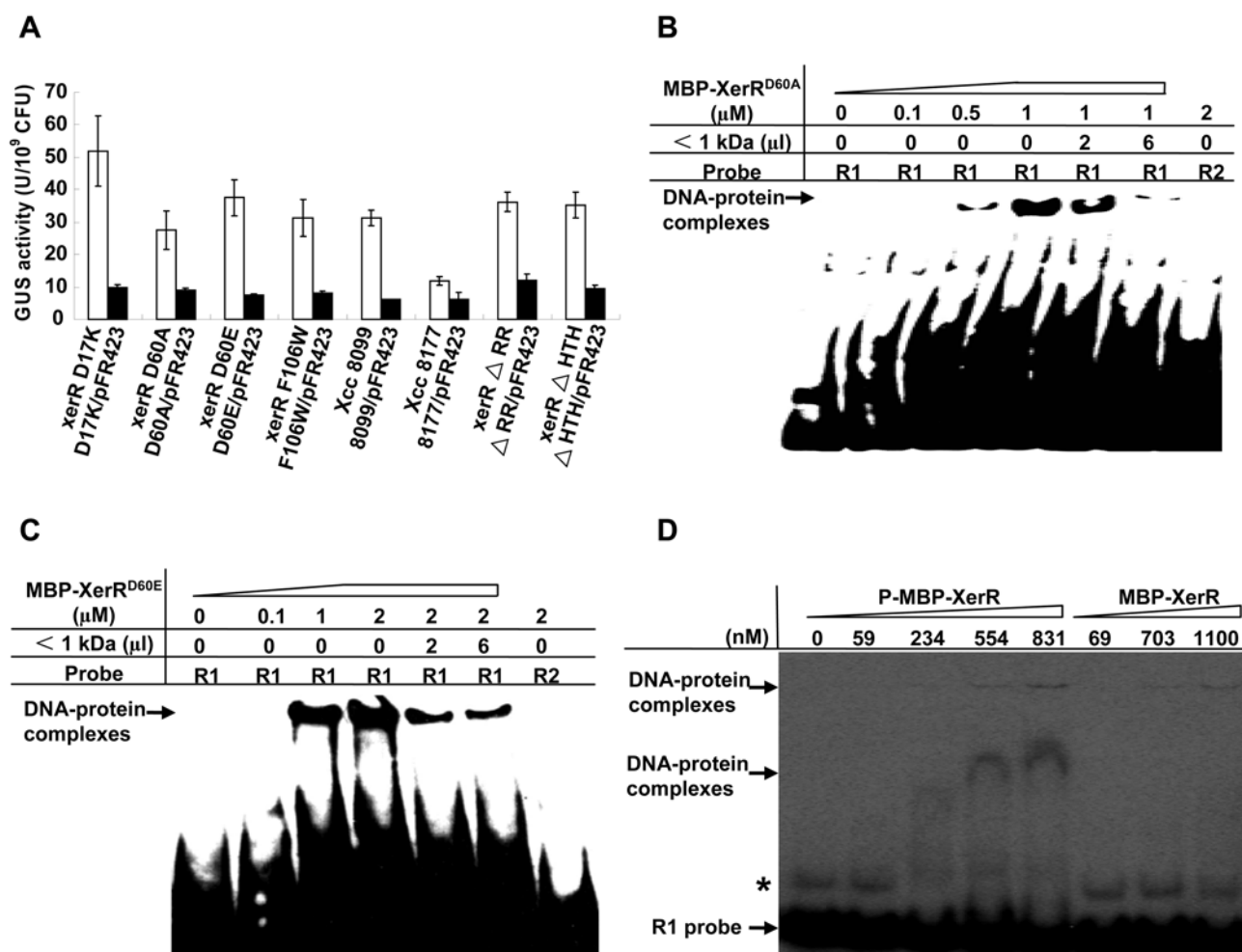


Figure 3 Phosphorylation-related residues of XerR are essential for its repressor function. **(A)** The conserved phosphorylation-related residues and the regulatory domains of XerR were indispensable for regulation of *xccR* expression *in vivo*. *xerR* Δ RR, *xerR* Δ HTH and different site-directed mutants exhibited increased GUS activities when grown in NYG medium. Plasmid-containing (pFR423) strains of different mutants reduced the GUS activities compared with that of *Xcc* 8177. Bacteria cultured in NYG medium were assayed at an OD₆₀₀ of 2.0. The experiments were repeated eight times with similar results. **(B and C)** EMSA assays of MBP-XerR^{D60A} and MBP-XerR^{D60E} with biotin-labeled R1 probe and plant extract. The two proteins presented the same binding characteristics to R1 probe, in which the plant extract of molecular weights < 1 kDa released the protein and DNA interactions. In the diagram, the concentration of purified protein and the volumes of plant signal(s) are indicated. **(D)** EMSA binding of phosphorylated and unphosphorylated MBP-XerR to the R1 probe. MBP-XerR was phosphorylated *in vitro* with acetyl phosphate and the R1 probe was end-labeled with ³²P at its 5' termini. The bands marked with an asterisk indicate a possible higher structure of R1 probe formed during annealing step.

thus de-represses the *xccR* transcription.

Next, we investigated the roles of the phosphorylation-related residues Asp-17, Asp-60 and Phe-106 in the repressor function of XerR. We constructed four *xerR* site-directed mutants on the *Xcc* 8177 background (*xerR* D17K, *xerR* D60A, *xerR* D60E and *xerR* F106W) and assayed their GUS activities (Figure 3A). Similar to the *xerR* null mutant *Xcc* 8099, each site-directed mutant showed a considerably higher GUS level than *Xcc* 8177, suggesting that the canonical phosphorylation-related residues are required for XerR function *in vivo*. Furthermore, the low-copy plasmid carrying wild-type *xerR* (pFR423) was able to restore the XerR repressor activity *in trans* in all of the *xerR* deletion and site-directed mutants (Figure 3A).

In addition, we analyzed whether the Asp-60-mutated proteins MBP-XerR^{D60A} and MBP-XerR^{D60E} can still bind the *xccR* promoter sequences, since Asp-60 was proposed to be the phosphorylation site by Pfam alignment. We found that although both mutated proteins could bind to R1, they lost the ability to bind R2 even at higher protein concentrations (Figure 3B and 3C), suggesting that XerR^{D60A} and XerR^{D60E} have altered DNA binding properties and thus cannot repress the *xccR* promoter.

On the other hand, we found that phosphorylation of XerR enhanced the binding to R1. As shown in Figure 3D, *in vitro* phosphorylated XerR (P-MBP-XerR) exhibited an affinity to bind R1 in EMSA at a concentration of 554 nM, which is lower than that needed for unphosphorylated MBP-XerR protein. Under an equivalent condition, we did not observe the band-shift at 1.1 μM for unphosphorylated MBP-XerR protein.

Taken together, the above results indicate that phosphorylation of XerR is essential for its repressor function, reminiscent of the intrinsic property of an NtrC family protein.

Inhibition of xccR expression by XerR is relieved in planta

We previously reported that the expression of *xccR* and *pip* was induced when the *Xcc* cells grew in the host cabbage [20]. In this report, we showed that XerR inhibited the expression of *xccR* and *pip* in culture medium. To see if the XerR-mediated inhibition is affected *in planta*, we quantified and compared the *xccR* expression levels *in planta* (Figure 4A) and in NYG medium (Figure 1B) in different *Xcc* strains. Overexpression of XerR in *Xcc* 8099/pFR423 and *Xcc* 8177/pFR423 greatly reduced the *xccR* promoter-directed GUS activities in NYG medium compared with that of *Xcc* 8177, as shown in Figure 1B. However, the GUS activities in the XerR-overexpressing strains were not reduced or even increased relative to that of *Xcc* 8177 when the bacteria grew *in planta* (Figure

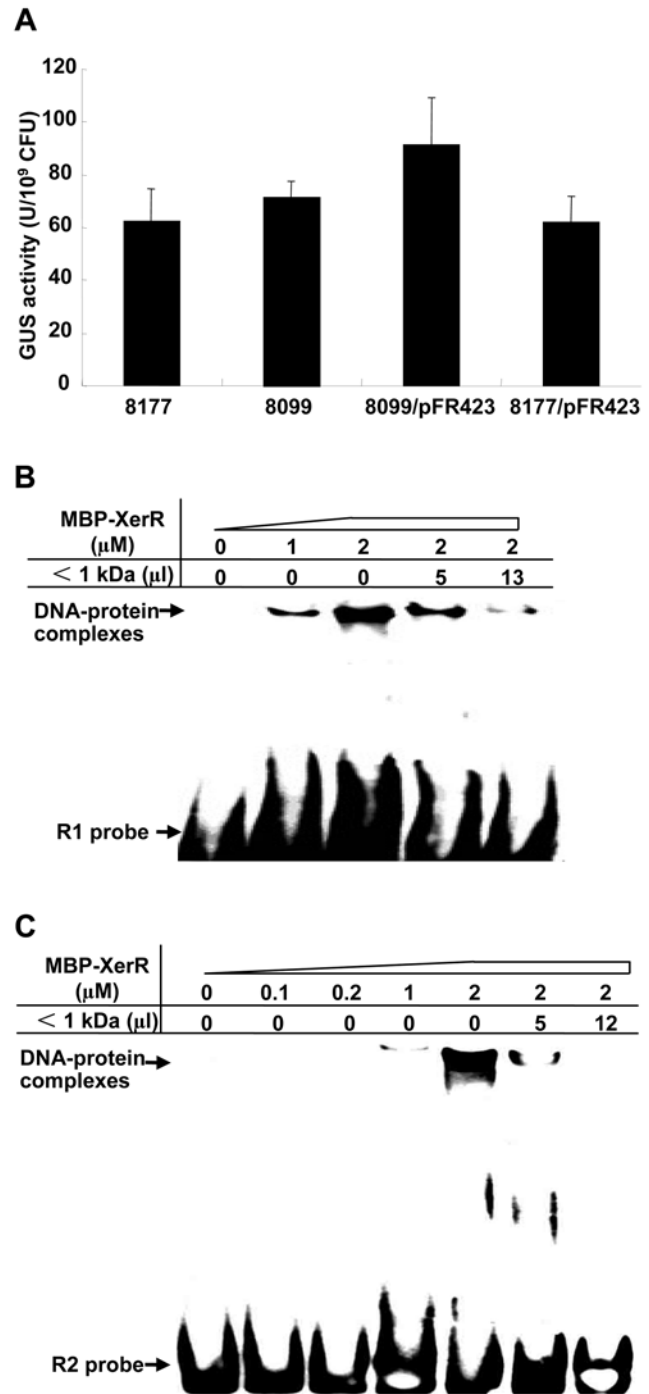


Figure 4 XerR relieves its inhibition on *xccR* expression *in planta*. **(A)** *In planta* cultivation did not significantly increase the GUS activity from *Xcc* 8099, while it had the opposite influence on that of *Xcc* 8099/pFR423 and *Xcc* 8177/pFR423. The bacteria were recovered from vacuum-infiltrated cabbage leaves 30 h post infiltration, and GUS activities were assayed. Data and standard deviation represented the mean of three independent measurements. **(B and C)** Plant signal(s) alleviated the binding activity of XerR protein to the *xccR* upstream regulatory sequence. EMSA assays with biotin-labeled probe were performed by MBP-XerR with plant extracts (< 1 kDa) at two dilutions.

4A). Furthermore, the *Xcc* 8099 strain had almost equivalent GUS activity as *Xcc* 8177 (Figure 4A), suggesting that the repression action of XerR on *xccR* expression might be relieved *in planta*.

As *pip* expression is controlled by XccR, we expected the inhibition of expression of *pip* by XerR in culture medium would also be relieved *in planta*. This was actually the case. As seen in Figure 1D, the significantly reduced *pip* transcript levels in XerR-overexpressing strains *Xcc* 8098/pFR423 and *Xcc* 8004/pFR423 in culture medium were restored to 63% and 90% of that of wild-type *Xcc* 8004, respectively, when the bacteria grew *in planta*. We reasoned that the increased expression of *xccR* and *pip* was not a result of reduced transcription of *xerR* gene in host plant, because the GUS activity of *xerR*-P/*gusA* *in planta* was 2.56-fold higher than that in medium alone (data not shown). In addition, the expression of *XC_3756*, another gene that is directly regulated by XerR via binding to the σ^{54} *cis*-element in its promoter, was enhanced threefold *in planta* compared with that in medium (data not shown).

To examine whether the observed de-repression of *xccR* expression was caused by plant factors that affected the binding of XerR to the *xccR* regulatory sequences, we performed EMSA in the presence of a low-molecular-weight (< 1 kDa) cabbage extract. As shown in Figure 4B and 4C, the presence of the plant extract disrupted the binding of XerR to the *xccR* promoter probes R1 and R2 in a dose-dependent manner. We infer from this result that some small molecules present in this cabbage extract are responsible for limiting the XerR binding to DNA. This interfering effect was DNA sequence specific, as the same amount of the cabbage extract did not cause dissociation of XerR from the promoter of another downstream gene *XC_3756* (data not shown). We further showed that the same extract also abrogated the ability of the mutant proteins MBP-XerR^{D60A} and MBP-XerR^{D60E} to retard the migration of the DNA probe R1 (Figure 3B and 3C), indicating that the two amino acid substitutions for Asp-60 of XerR do not alter the interaction between XerR and the plant signal(s).

Cabbage extract enhances the binding of XccR to the *pip* promoter

We previously reported that in a super-shift assay, a cabbage ethanol extract enhanced the binding of XccR to the *pip* promoter [20]. In addition, it was reported that an unknown rice signal molecule present in the < 1 kDa fraction of an *Xoo*-infected rice extract increased the solubility of OryR [21]. We thus wanted to see if the < 1 kDa cabbage extract, which abolished the binding of XerR to the *xccR* promoter, could affect the binding of

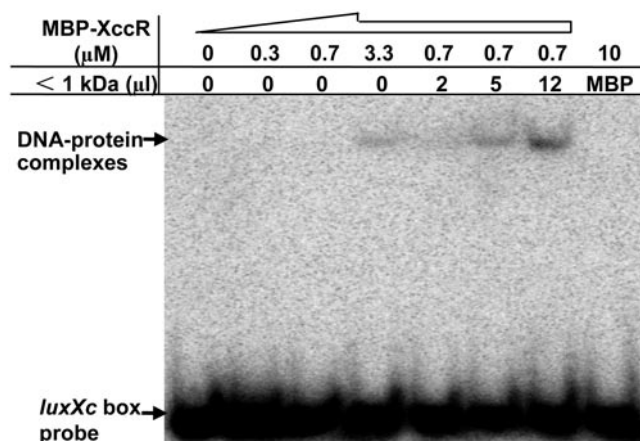


Figure 5 EMSA binding of MBP-XccR protein to the *luxXc* box of the *pip* promoter. The band of XccR and DNA complex was intensified by adding different volumes of plant extracts in the EMSA assay. The migrated DNA-protein complexes and isotope-labeled *luxXc* box probe are indicated by arrows.

XccR to the *luxXc* box of the *pip* promoter. We found that the < 1 kDa extract indeed stimulated the binding of MBP-XccR to the *luxXc* box sequence at an MBP-XccR concentration of 0.7 μM at which no protein-DNA binding occurred without the plant extract (Figure 5). Moreover, the formed protein-DNA complex was significantly intensified with an increase in the concentration of the plant extract (Figure 5). The above results indicate that the same cabbage extract shows different effects on the formation of the XerR/*xccR* promoter complex and the XccR/*pip* promoter complex. Although we cannot conclude that XerR and XccR interact with the same compound in the cabbage extract, our results portray a subtle regulatory pattern in which *Xcc* recruits plant signal(s) to sequester XerR from its binding sequence, yet on the other hand, to stimulate the XccR binding to the *pip* promoter for infectivity.

Discussion

In Gram-negative bacteria, LuxR/LuxI is the most well-defined regulatory system that modulates gene expression related to QS. This system can monitor the concentration of AHL-like small molecules in the environment and control downstream gene expression or cell behavior [34, 35]. In *Xanthomonas*, the solo (orphan) LuxR homologs, including XccR from *Xcc* and OryR from *Xoo*, can sense chemical signals derived from host plants and take part in bacterial pathogenesis by regulating the expression of virulence factors [20-22]. These studies strongly suggested an interesting phenomenon

that an inter-kingdom communication exists between phytopathogens and their host plants. However, how bacteria sense signals from plant and the nature of the plant signal(s) remain unclear.

In this study, an NtrC family RR was identified by genome-scale screening with a transposon insertional mutant library. By measurement of GUS activity, XerR was confirmed to be a negative regulator of *xccR* expression (Figure 1B). XerR can bind R1 and R2 regions of *xccR* upstream sequence (Figures 2B and 4C). It was shown that mutations of the conserved phosphorylation-related sites on XerR resulted in upregulation of *xccR* expression (Figure 3A) and that *in vitro* phosphorylated XerR showed enhanced affinity to the R1 probe (Figure 3D), both results suggesting that protein phosphorylation is required for the repression function of XerR. In addition, GUS assays *in planta* (Figure 4A) as well as EMSA experiments (Figure 4B and 4C) showed that the binding of XerR to the *xccR* upstream DNA sequence was substantially inhibited in the presence of the plant extract, suggesting that a plant signal(s) modulates the *xerR/xccR/pip* regulatory cascade.

By secondary protein structure prediction (Figure 1A), XerR was found to be a typical NtrC-family RR of the bacterial TCSTS. It contains an N-terminal CheY-like receiver domain, a C-terminal HTH domain, and a central σ^{54} interaction domain responsible for the initiation of an open transcriptional complex [36]. In prototypical TCSTS, a histidine kinase (HK) sensor can monitor specific environmental stimuli. After autophosphorylation on a conserved histidine residue, the dimeric HK transfers the phosphoryl group onto the conserved asparagic acid residue of the cognate RR, and the latter will regulate downstream gene expression, usually acting as a transcription factor [37, 38]. As mentioned above, site-directed mutations, which changed the three critical sites (Asp-17, Asp-60 and Phe-106) related to protein phosphorylation, nearly abolished the repressor activity of XerR (Figure 3A). In addition, XerR can receive the phosphorylation signal *in vitro* (Figure 3D). These results suggest that under the culture conditions, an unidentified HK can phosphorylate XerR specifically and cause it to bind to the *xccR* promoter with high affinity. Transcriptional repression of *xccR* may result from the direct competition of phosphorylated-XerR and RNA polymerase on its promoter or the blocking of mRNA elongation, as the probes that we used spanned from -99 to +9 bp relative to the *xccR* translational start site. The repression mechanism of XerR remains to be fully elucidated. Intriguingly, XerR^{D60A} and XerR^{D60E} abolished the binding ability to R2 (Figure 3B and 3C), whereas the *E. coli* NtrC^{D60A} and NtrC^{D60E} proteins bound to the cognate

enhancer sequence normally [39, 40].

In addition, our results also showed that the repressor function of XerR was relieved when *Xcc* was grown within the host plant (Figure 4A). In TCSTS, the central mechanism is the balance of phosphorylated and dephosphorylated RR [41, 42]. Since the majority of HKs also have phosphatase activity to dephosphorylate RR [43-45], we speculated that the cognate HK of XerR *in planta* may exhibit phosphatase activity, rather than acting as a kinase, which would result in the dephosphorylation of XerR and thus decrease its affinity in binding the *xccR* promoter. Because there is no HK gene located in the vicinity of *xerR*, it is extremely difficult to identify its cognate HK only by bioinformatic analysis. Currently we have identified a candidate HK gene that also exhibits repressor function in regulating *xccR* transcription (unpublished data). Further investigation will clarify whether it is the cognate HK of XerR and how it modulates the phosphorylation state of downstream RR when *Xcc* survives in different ecological niches.

Besides phosphorylation-regulated *xccR* expression via classical TCSTS, our experiments also suggest that the activity of XerR is affected by chemical signal(s) from host plants. As shown in Figure 4B and 4C, when the plant extract was present in EMSA, the binding between XerR and the *xccR* promoter was disrupted in a dose-dependent manner. We found that the expression of *xerR* was increased *in planta*, and XerR-mediated upregulation of the transcription level of *XC_3756* was not affected when *Xcc* was grown in the host plant, implicating that the unidentified plant chemical(s) has a specific influence on the binding between XerR and the *xccR* promoter. Inter-kingdom communications involved in de-repressing the expression of a bacterial gene by plant signal(s) also occur in other bacterial-eukaryotic systems. For instance, the repression of pectinase genes by the transcriptional repressor KdgR in *Erwinia carotovora* was abolished in the presence of plant cell wall breakdown products, and agrocinopines de-repressed the AccR binding activity to the *arc* operon in *A. tumefaciens* [46-48]. Intriguingly, the same plant extract improved the binding of XccR to the *luxXc* box of the *pip* promoter, indicating that XerR and XccR may simultaneously interplay with plant signal(s) in regulation of the *xccR/pip* locus, imposing a strict control on the expression of a virulence gene.

It was shown that host plant signal(s) with molecular weights less than 1 kDa inhibited the repression function of XerR in a concentration-dependent manner (Figure 4B and 4C). Although most of the small signaling molecules to date were extracted by organic solvents, including furanones, flavonoid, riboflavin and its derivative lu-

microbe [49-52], our cabbage extract was water based. We propose that the signal(s) may be peptides, amino acid or its derivatives, monosaccharides, oligosaccharides, aminosugars, aminoglycosides or acid and alkali compounds. It has been reported that two free amino acids, homoserine and asparagine, act as host signals inducing the *pelD* expression of *Nectria haematococca* in pea seedlings [53].

In brief, in the interaction between *Xcc* and the host plant, *Xcc* builds up a sophisticated mechanism in the *xerR/xccR/pip* pathway. In this pathway plant signal(s) either activates *XccR* to positively regulate *pip* transcription or relieves the inhibition of *XerR* on *xccR* expression. As illustrated in Figure 6, we propose a model that the transcriptional inhibitory role of *XerR* is regulated by an unknown HK, and *XerR* then blocks the transcription of *xccR* by directly binding to the *xccR* upstream sequence during growth in the medium. When *Xcc* grows in the host plant, the conformational changes of *XerR* and *XccR* induced by the plant small molecule(s) lead to the release of *XerR* from the *xccR* promoter and the increase in the binding of *XccR* to the *luxXc* box. Consequently, the expression of the *pip* gene, which plays a crucial role in bacterial pathogenesis, is fine-tuned in the host plant.

Materials and Methods

Strains and reagents

The wild-type *Xcc* strain 8004 and *Xcc* 8177 harboring a chromosomal *xccR-P/gusA* fusion in *Xcc* 8004 were described previously [20, 54]. *Xcc* strains were routinely cultivated at 28 °C in NYG medium, whereas *E. coli* strains were grown aerobically at 37 °C in LB medium. Antibiotics were added at the following concentrations: for *Xcc*, rifampicin (100 µg/ml), spectinomycin (150 µg/ml) and kanamycin (100 µg/ml); for *E. coli*, spectinomycin (150 µg/ml), kanamycin (50 µg/ml) and ampicillin (100 µg/ml). The reagents 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc), 4-methylumbelliferyl-β-D-glucuronide (MUG), 4-methyl-umberliferone and lithium potassium acetyl phosphate were purchased from Sigma. RNase-free DNase, M-MLV Reverse Transcriptase and Random Primers were from Promega and the Lightshift Chemiluminescent EMSA kit was from Pierce.

Creation of *Xcc* mutants and preparation of *in trans* expression constructs

Deletion strains were generated with the suicide vector pK18*mobSacB* [55] by a long-flanking homology procedure and two-step recombination [56]. All DNA manipulations were performed according to standard procedures. Plasmid DNA was transferred to *E. coli* by heat shock and to *Xcc* strains by electroporation. Unless otherwise specified, the corresponding gene fragments were PCR amplified and first cloned into pEASY-T1 Vector (TransGen Biotech) for sequence verification. After digestion with appropriate enzymes, these fragments were cloned into corresponding vectors to generate the constructs used in this study.

To construct *Xcc* 8098, two ~500-bp sequences upstream and downstream of the *xerR* reading frame were amplified by PCR. The in-frame deletion resulted in removal of the codons for amino acid residues 8 to 428. After digestion with appropriate enzymes, a pK18*xerR* clone was created by cloning two recovered fragments into pK18*mobSacB* simultaneously. The pK18*xerR* plasmid

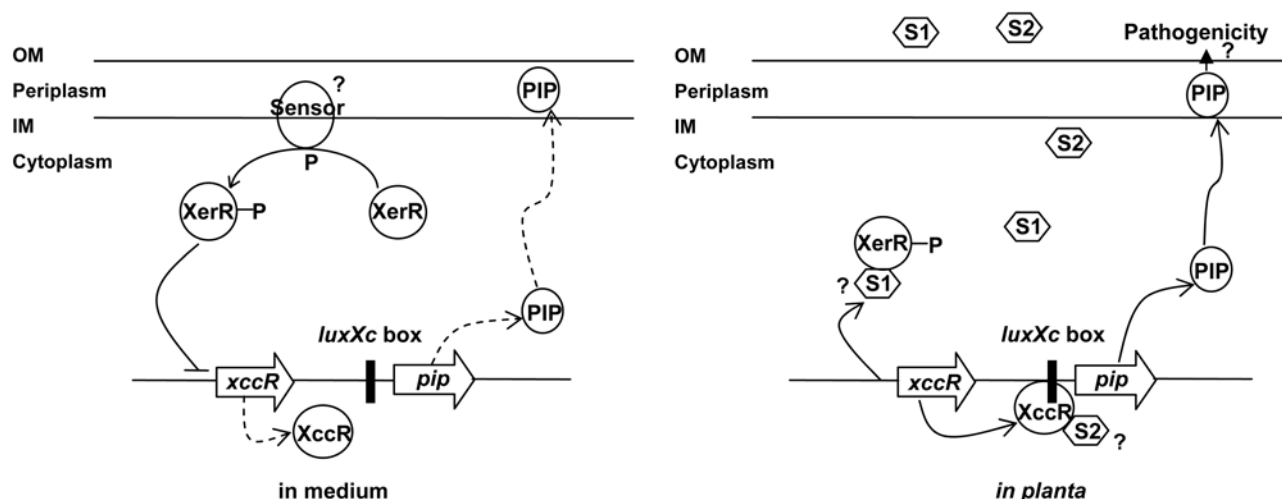


Figure 6 A model for expression regulation of *xccR/pip* locus by *XerR*. When *Xcc* is grown in NYG medium, *XerR* represses the expression of *xccR* and *pip*. The activity of *XerR* is dependent on the phosphorylation by an unknown two-component signaling transduction system. After entering the host plant, *XerR* relieves its inhibition to *xccR* expression in response to specific plant small molecule(s). The released *XccR* binds to *luxXc* box in the presence of the same or different plant signal(s) to induce the transcription of the *pip* gene for bacterial virulence. S1 and S2 denote the possible signal(s) from the host. OM and IM refer to the outside and inside membrane, respectively.

conferring kanamycin resistance (Kan^R) and sucrose sensitivity (Suc^S) from white colonies was verified by restriction digestions or by sequencing, and then transferred to *Xcc*. Allelic replacement was achieved by sequential selections on kanamycin and 10% sucrose to create *Xcc* 8098. Positive transformants of *Xcc* 8098 were confirmed by PCR and sequencing. To introduce *xccR*-P/*gusA* into *Xcc* 8098, a 3.2-Kb DNA fragment carrying the *xccR* promoter and the *gusA* gene was cloned into pK18*mob* vector to generate plasmid pFR435. The suicide vector pFR435 was integrated into the chromosome of *Xcc* 8098 by homologs recombination via a 542-bp sequence of the *xccR* promoter. The resultant strain was termed *Xcc* 8099. PCR was used to identify positive transformants, and the PCR products were sequenced. The same procedures were applied in generation of the other two deletion mutants: *xerR* ΔRR and *xerR* ΔHTH.

To perform *in trans* expression analyses, the entire coding sequence of the *xerR* gene was PCR-amplified and constructed into pHM1, a broad host range expressing plasmid [57]. The generated clone was sequence verified and named pFR423. pFR423 was then electrotransformed into *xerR* null mutant 8098, 8099 and *Xcc* 8004, resulting in the complemented and overexpressed strains *Xcc* 8098/pFR423, *Xcc* 8099/pFR423 and *Xcc* 8004/pFR423, respectively. The resultant transformants were selected on NYG medium supplemented with rifampicin and spectinomycin. Plasmid derivatives harboring the correct inserts in *Xcc* strains were extracted and verified by restriction digestions.

Site-directed mutagenesis

Three conserved amino acid residues of XerR (Asp-17, Asp-60 and Phe-106, in the RR domain) predicted to be involved in phosphorylation were identified via the sequence alignment with the homologs: NtrC (NCBI accession number X85104), CheY (M13463) and LuxO (L26221). A 753-bp fragment containing part of the *xerR* gene and its flanking sequence was PCR-amplified and cloned into pEASY-T1, resulting in pEASY-T753. Three conserved residues were changed to lysine (D17K), alanine (D60A), glutamate (D60E) and tryptophan (F106W) by site-directed mutagenesis (Easy Mutagenesis Systems, TransGen Biotech) using the pEASY-T753 vector as the template. The four resultant 753-bp mutant fragments were separately inserted via *Spe*I and *Pvu*II sites into pK18*xerR*-28, a plasmid containing the *xerR* gene and its up- and down-stream flanking sequences in pK18*mobSacB*. By homologs recombinations, the mutation-containing plasmids pK18*xerR*-D17K, pK18*xerR*-D60A, pK18*xerR*-D60E and pK18*xerR*-F106W were individually incorporated into *Xcc* 8098. Positive clones were verified by PCR and DNA sequencing. The same homologs recombination procedures were used to insert the *xccR*-P/*gusA* cassette into different mutants to create *xerR* D17K, *xerR* D60A, *xerR* D60E and *xerR* F106W.

GUS assay

GUS assays were used to examine the expression of the *xccR*-P/*gusA* fusion in different *Xcc* strains. GUS activity of the bacteria grown in medium and *in planta* was measured by the fluorometric method using MUG as a substrate essentially as described in Zhang *et al.* [20].

RNA extraction and RT-PCR analysis

Bacterial cells at OD₆₀₀ of 1.5 to 2.0 were harvested by centrifu-

gation at 4 °C for 2 min at 12 000× *g*. Total bacterial RNA was isolated using TRIzol reagent (Invitrogen) following the protocol provided by the manufacturer. RNase-free DNase I was used to treat the RNA samples. RT was performed using M-MLV Reverse Transcriptase with random hexadeoxynucleotides as primers. Typically, 25 ng of cDNAs was used for each PCR reaction in a 25 μl mixture. The 16S rRNA was served as an RT-PCR internal reference.

To assay the level of the *pip* transcript in *Xcc* strains grown *in planta*, *Xcc* cells were vacuum infiltrated into cabbage seedlings as described by Zhang *et al.* [20]. Plant leaves harvested at 30 h post infiltration were homogenized in liquid nitrogen, and RNA isolation and RT-PCR were performed as described above. RNA from un-infiltrated leaves was used as a control.

Protein expression and purification

Prokaryotic expression plasmids pMX766 (pMal-p2X(*lac*-P/*xerR*)), pMX767 (pMal-p2X(*lac*-P/*xerR*-D60A)), pMX768 (pMal-p2X(*lac*-P/*xerR*-D60E)), pMX769 (pMal-p2X(*lac*-P/*xccR*)) were transformed into *E. coli* TB1. Two milliliters of the overnight culture were inoculated into 200 ml LB broth plus 2% glucose and ampicillin. After the cells were grown at 37 °C to an OD₆₀₀ of 0.5, MBP-fusion proteins were induced by addition of IPTG to a final concentration of 0.3 mM and cell growth was continued overnight at 16 °C with a gentle shaking at 180 r.p.m. The cells were harvested, and the soluble MBP-tagged proteins were purified by affinity chromatography with amylose resin (BioLabs). Briefly, each cell pellet was resuspended in 10 ml of the column buffer (20 mM Tris-HCl, pH 8.0; 200 mM NaCl; 1 mM EDTA) plus 1 mM PMSF, incubated on ice for 20 min, sonicated, centrifuged and the supernatant was added to 2 ml of amylose resin slurry. After washing six times with the column buffer, the proteins were eluted using 10 ml of the column buffer plus 10 mM maltose three times. The purified protein samples were combined, and the solvent was changed to a buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 5% glycerol. Finally, the proteins were concentrated to approximately 1-10 mg/ml using Amicon YM-10 column (Millipore) and filtered through an Ultrafree-MC (0.45 μm) spin filter (Millipore) before aliquoting for storage at -80 °C. Protein concentrations were measured using the Bio-Rad Protein Assay reagent with BSA as a standard. About 5 μg of each protein sample was analyzed by 8% SDS-PAGE to verify molecular weight and purity.

Electrophoretic mobility shift assay

MBP-XerR and MBP-XccR fusion proteins were purified through amylose columns as described above. Four 59-nt single-stranded DNA oligonucleotides containing putative XerR-binding sequences upstream of the *xccR* coding region and two 46-nt *pip* promoter sequences with or without biotin labeling were synthesized by Invitrogen. DNA duplexes required for EMSA were annealed by mixing equal amounts of single-stranded oligos and incubating the mixture for 10 min at 93 °C in annealing buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA; 100 mM NaCl). After slowly cooling down for 2 h at room temperature, the annealed probes were aliquoted for storage at -20 °C and thawed on ice before use. The isotope-labeled probe was end-labeled by using (α-³²P)-dATP (PerkinElmer) and the Klenow fragment of DNA polymerase I (Promega). The labeled probe was purified with Sephadex G-50.

Binding reaction mixtures contained 20 fmol of the DNA biotin-labeled probe, various amounts of MBP-XerR or MBP-XccR protein in a buffer of 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 50 ng/ μ l poly(dI-dC) DNA in a volume of 20 μ l. After a 30-min incubation at room temperature, 4 μ l of 80% glycerol was added to each reaction and samples were size fractionated using 5% polyacrylamide gels in 0.5 \times TBE buffer (45 mM Tris-borate; 1 mM EDTA) at 4 $^{\circ}$ C. For competition, a certain amount of unlabeled probe or plant signal(s) was co-incubated with the protein for 20 min at room temperature before adding labeled probe. The reaction samples were electrophoretically transferred to a nylon membrane (Hybond-N⁺, Amersham Biosciences) using wet transfer, and then the membranes were crosslinked by a UV lamp at 120 mJ/cm². Detection of biotin-activated light signals was performed according to the manufacturer's instructions described by the LightShift Chemiluminescent EMSA Kit (Pierce).

For EMSA using isotope-labeled probe, 8 fmol of labeled probe was added to the mixtures. The reaction procedure and electrophoresis were the same as described above. The gel was dried and subjected to autoradiography.

Phosphorylation of MBP-XerR protein

Phosphorylation of the purified MBP-XerR protein was performed essentially as described previously [58, 59]. Briefly, ~50 μ g of MBP-XerR was incubated with 50 mM acetyl phosphate (lithium, potassium salt, from Sigma) for 1 h at 30 $^{\circ}$ C in a buffer of 100 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 125 mM KCl. The concentration of the phosphorylated protein was measured, and the conditions for using it in EMSA were the same as described above. In parallel, similar reactions lacking acetyl phosphate were used to prepare MBP-XerR for EMSA studies.

Preparation of low-molecular-weight plant extracts

About 20 g fresh cabbage leaves were homogenized in liquid nitrogen, and the powder was resuspended in 100 ml water. The extract was centrifuged, fractionated in series by 0.45 μ m filter membrane, ultrafiltration membranes YM10 and YM1 to obtain the compounds of molecular weights less than 1 kDa.

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