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Western Blot Detection of Xanthomonas Oryzae pv. Oryzae in Rice

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Abstract

Bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is a severe rice disease, and rice-*Xoo* interactions form a model system in plant disease resistance research. To establish immuno-techniques for *Xoo* detection, a *Xoo*-specific polyclonal antibody was generated using the total *Xoo* protein as the immunogen. A western blot procedure, which can detect as few as 3,500 *Xoo* cells, was established. *Xoo* can be detected in rice at 1-day post-inoculation (dpi) and a significant difference between the number of *Xoo* cells in resistant and susceptible reactions was found at 2 dpi. In the incompatible rice-*Xoo* interaction, *Xoo* can be detected within 6 cm of the inoculated site at 10 dpi. In the compatible interaction, *Xoo* can be detected within 12 cm of the inoculation site, while no *Xoo* signal was detected beyond that area in inoculated leaves and uninoculated leaves, supporting the hypothesis that rice plants harboring resistance genes have stronger inhibitory effects on *Xoo* propagation. This method is expected to replace the traditional manner of counting bacterial cells in laboratories and of inspecting *Xoo*-infected rice in paddy fields.

Keywords: Rice; *Xanthomonas oryzae* pv. *oryzae* (*Xoo*); Bacterial blight; Western blot; Antibody

Introduction

Rice is an important food crop, being the staple of more than half of the world's population. Bacterial blight caused by the Gram-negative bacteria *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is the most severe rice disease, causing significant yield losses worldwide [1]. The bacterium invades xylem tissue, either through wounds or water pores, leading to systemic infection [2,3]. In the past decades, a number of resistance genes from rice and avirulent genes from *Xoo* have been cloned. The whole genome sequencing of both rice [4] and *Xoo* [5] facilitated using the rice-*Xoo* model system to study plant-microbe interactions [6].

The detection of pathogens is critical to ensure high and stable agricultural yields. For rice bacterial blight, experienced breeders can evaluate the degree of infection based on visual inspection. In laboratories, the common detection method involves clipping the leaves, culturing the extract on bacterial medium, and then quantifying the number of *Xoo* based on colony-forming units [7]. A similar method for counting bacterial cells was established in *Arabidopsis thaliana* [8]; however, this method is time consuming, taking several days, and labor intensive.

PCR-based DNA amplification is also used in the detection of *Xanthomonas* pathogens [9,10]. Because the whole genome sequence is available, *Xoo*-specific primers can be designed to differentiate it from similar species [11]. In addition, gene-specific primers can also be used for PCR amplification [12]. PCR is also able to detect *Xoo* in rice at different time points and positions [13,14].

A number of modified PCR versions, such as Padlock and LAMP, have also been used to detect *Xoo* in rice [15,16]. The amplification reactions in these methods were carried out at a constant temperature, and the products can be visualized. Such methods are expected to be used for *Xoo* detection in paddy fields. Plant pathogen detection using PCR is convenient and highly sensitive; however, false-positive results occur frequently [15]. Unless real-time PCR is used, regular PCR cannot detect pathogens quantitatively, thus it is difficult to monitor *Xoo* propagation in rice.

Immunological assays, which are also convenient, as well as specific and sensitive, were widely developed for the detection of pathogens in humans and animals [17]. The detection of pathogens in the genus of *Xanthomonas* using immunological assays was first reported in the last century [18-21]. Recently, hairpin protein-specific antibodies of *Xoo* were generated and used to detect transgenic rice [22,23]. Nonetheless, an immunoassay to detect *Xoo* in rice has not been reported.

In recent years, fluorescent proteins have been used to monitor living organisms, such as Pseudomonas syringae, X. axonopodis pv. dieffenbachiae, and Xylella fastidiosa, in their hosts [24,25]. Applications of fluorescent marker systems can facilitate the detection of invading pathogens, and monitor the migration and proliferation of the bacteria. Green fluorescent protein (GFP) was introduced into Xoo to assess bacterial infections and multiplication in planta [26]. With this fluorescent marker system, the bacterial population can be measured in a day, and resistant/susceptible lines can be screened at 4 days postinoculation (dpi). Using a Xoo strain expressing GFP (Xoo-GFP), rice plants harboring XA21 can restrict the spread of Xoo from the point of infection. Moreover, the spatial distributions of Xoo populations in planta can be measured quantitatively [27]. However, the application of the fluorescent protein-based approach was limited to a specific mutant, and its virulence and pathogenicity required approval. Under certain circumstances, the auto-fluorescence from rice plants may perturb the signal intensity from the bacterial population.

In this study, a *Xoo*-specific polyclonal antibody was generated, and a western blot (WB) procedure was established for the quantitative detection of *Xoo* in rice. *Xoo* could be detected within 1 dpi. Moreover, incompatible and compatible interactions could be distinguished at 2 dpi. Thus, the WB operation is convenient and usually completed within 1 day. This method is expected to substitute the procedure of counting bacterial cells in laboratories and to inspect *Xoo* infected rice in paddy fields.

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Materials and Methods

Plants and bacteria

TP309 is a *japonica* rice cultivar (*Oryza sativa* L.), and 4021 is a homozygous transgenic line of TP309 harboring the bacterial blight resistance gene *Xa21* [28, 29]. Both lines were cultivated at an experimental plot in the west campus of Hebei Agricultural University (Baoding, Hebei, China). TP309 shows a susceptible reaction (S) with *Xoo* race P6 (PXO99Az), while 4021 shows a resistant reaction (R) with P6 [30]. P2, P5, P6, P8, and P9 stains of *Xoo* and *Magnaporthe oryzae* stain TH12 were provided by Drs. Wen-xue Zhai and Guanghuai Jiang from the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences. The DH5a stain of *Escherichia coli* was saved in our laboratory. *Xoo* was cultivated on PSA (0.5% Bacto peptone, 2% sucrose and 0.05% L-glutamic acid) plates at 28°C [31]. *Magnaporthe oryzae* was grown on medium (0.6% yeast extract, 0.6% peptone, 1% sucrose and 1.5% agar) at 25°C under a 16-h/8-h (light/ dark) photoperiod.

Rice inoculation and sample collection

Xoo was cultured on PSA medium for 48-72 h, collected using sterilized distilled water, and then diluted to an OD_{600} equal to 1. Inoculations were carried out using sterilized scissors dipped into the bacterial liquid to cut leaves. Healthy leaves from rice plants at the tillering stage were inoculated. Rice samples were collected at the time points 0 h, 1 day, 2 days, 3 days, 4 days, 5 days, 7 days, 10 days, and 15 days after inoculation. Moreover, the upper (within 6 cm of the inoculation site), middle (6-12 cm from the inoculation site) and lower (12-18 cm away from the inoculation site) parts, as well as the uninoculated leaves of the same plant, were collected at 0 h, 3 days, 5 days, and 10 days after inoculation. The samples were frozen in liquid nitrogen immediately after collection and then stored at -70°C until use.

Total protein isolation

Rice samples were ground into a fine powder in liquid nitrogen and dissolved in extraction buffer [62.5 mmol L-1 Tris-HCl (pH 7.4), 10% glycerol, 0.1% SDS, 2 mmol L⁻¹ EDTA, 1 mmol L⁻¹ PMSF, and 5% β-mercaptoethanol]. The mixture was vortexed thoroughly and then chilled on ice for 10 min. Cell debris was removed by centrifugation at 12,000 \times g for 20 min at 4°C, and the supernatant was collected as a sample of rice total protein. A detailed protocol can be found in our previous report [32]. Xoo colonies growing on PSA plates were eluted using sterilized water and collected by centrifugation. The bacteria were dissolved in $4 \times \text{loading buffer}$ [50 mmol L-1 Tris (pH 6.8), 200 mmol L-1 DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol] and then heated in boiling water for 20 min. Supernatants were collected by centrifugation as the total protein of Xoo. In addition, a fraction of the Xoo cells was sonicated for 30 cycles at 500 W for 10s/15s (on/off; sonicator model: JY90-IIN, Xinzhi Biotechnique Company, Ningbo, Zhejiang, China), and then protein isolations were performed independently.

Counting of bacterial cells

The collected *Xoo* samples were diluted to an OD_{600} of 1 and then diluted further to 10⁵, 10⁶, and 10⁷. The diluted *Xoo* cells at different concentrations were spread on PSA plates. Colony counting was performed and the average was used to calculate the number of *Xoo* bacterial cells.

Production of polyclonal antibodies against Xoo and an ELISA test

A polyclonal antibody against Xoo was obtained by immunizing New Zealand white rabbits (weighing ~2 kg) with Xoo total protein. Briefly, the rabbits were initially immunized subcutaneously at two to four different sites with Xoo total protein (200-300 µg) dissolved in phosphate buffered saline (PBS, pH 7.4) and complete Freund's adjuvant in a 1:1 ratio. Then, two booster injections were performed in animals at 15-day intervals using the same amounts of antigen in incomplete Freund's adjuvant. The blood samples taken from marginal ear veins of the rabbits before immunization were used as negative controls. Bleeding (30 mL) was done 10 days after the last booster shot and then stored at 4°C overnight. Following centrifugation at $10,000 \times g$ at 4°C for 10 min, the serum was obtained by decanting the supernatant. The polyclonal antibody level in the serum was determined by the ELISA method. The obtained antibody-containing serum was stored in aliquots at -20°C for later use. The generation of the anti-Xoo antibody was performed by Beijing Protein Innovation Co., Ltd.

Procedures for the ELISA analysis were carried out briefly as follows: ELISA plates were coated with 100 µL of antigen at a concentration of 2 µg m L⁻¹ in sodium bicarbonate buffer (pH 9.6). The coated plates were incubated at 4°C overnight. After blocking at 37°C with phosphate buffer containing 1% skimmed milk (w/v) for 2 h, the plates were washed with phosphate buffer. Test sera were serially diluted with PBS and then added, in duplicate, to the plates and incubated for 1 h at 37°C. PBS buffer was used as the blank control, and serum collected before immunization was used as the negative control. After three washing steps with the phosphate buffer, bound antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit antibodies using 3'3'5'5'-tetramethylbenidine dihydrochloride (0.1 mg m L⁻¹ final concentration) plus 0.1% H₂O₂ in phosphate-citrate buffer. The color development was stopped by adding 50 µL per well of 2 N H₂SO₄. The optical density was recorded at 450 nm using a spectrophotometric microplate reader.

WB analysis and data collection

A detailed WB protocol was reported previously [32]. The heat shock protein (HSP) signal was used as the loading control for rice proteins [33]. The signal intensity was extracted using the SAGE Lane 1D Gel imaging analysis software (SAGE Company, Beijing, China). The averages and standard deviations were calculated based on signals from three repeats of WB analyses, and the relative intensity of each sample was compared.

Data normalization

To normalize data collected from different WB analyses, a specific number was given to the total intensity of the WB signal, and then the relative intensity was calculated accordingly. The average and standard derivations of three repeats were used to draw a standard curve and to obtain an equation of linear regression using Excel software (Microsoft).

Results

Xoo total protein isolation

Xoo cells were collected from PSA plates and total proteins were isolated. A quantitative analysis was carried out based on a reference [34]. SDS-PAGE separated total protein was stained using Coomassie blue. To determine the effects of cell disruption on protein extraction, we compared the proteins isolated from *Xoo* cells with or without

sonication. The Coomassie blue staining demonstrated that there were no significant differences between the two treatments.

Evaluation of the anti-Xoo antibody

Total proteins isolated from both X00-P6 cells with or without sonication were used as the immunogens to inject New Zealand white rabbits to generate polyclonal anti-Xoo antibodies. ELISA experiments were performed to evaluate the antibody titers using 96-well plates coated by immunogen (Figure 1). As Figure 1 shows, even at a 102,400 × dilution, the antibodies could still detect signals significantly higher than in the blank controls, and the titers of two antibodies were similar. However, the titer of the anti-Xoo antibody generated using protein isolated from sonicated Xoo cell (Ab-s) was higher than that generated from the non-sonicated sample, therefore, the Ab-s antibody was used in the experiments. To detect the specificity of the antibody, total proteins isolated from rice (TP309), E. coli (DH5a), Xoo (P2, P6, P8, P9, and P10), and M. oryzae (TH12) were separated on SDS-PAGE and assayed using a WB (Figure 2). The antibody could detect different races of Xoo and did not cross-react with rice, E. coli, or M. oryzae, indicating that the antibody is highly specific to the Xoo protein. In addition, several major bands were detected using a WB of the Xoo protein, supporting the hypothesis that multiple antigenic epitopes existed to produce the polyclonal antibodies.

Sensitivity and standard curve of the WB detection of Xoo

To test the sensitivity of the WB detection of *Xoo*, serially diluted samples containing different *Xoo* cell numbers were assayed using WB. The minimum number of *Xoo* cells detectable by WB was 3,500 (in 20 μ L loading buffer, ~1.75 × 10⁵ colony-forming units m L⁻¹). Within a certain range, there was a linear relationship between the signal intensity of WB and the number of *Xoo* cells (data not shown). We next detected the *Xoo* protein when mixed with rice samples (TP309) (Figure 3). The rice proteins did not interfere with *Xoo* protein detection, and there was a linear relationship, within a certain range, between the WB signal intensity and the *Xoo* cell number. The linear regression equation was: y=1.6422 × -3.0408, with a correlation coefficient (R²) of 0.9643. This result provided the groundwork for the detection of *Xoo in planta*.

Quantitative measurements of Xoo in planta

To detect Xoo in rice plants, Xoo race P6 was used to inoculate TP309 and 4021 using the leaf-clipping method. At 15 dpi, the lesion length caused by the TP309-P6 interaction was ~14 cm, indicating a typical susceptible reaction, and the lesion length caused by the 4021-P6 interaction was ~1 cm, indicating a typical resistant reaction (Figure 4). Leaf samples were collected at 0 h, 1 day, 2 days, 3 days, 4 days, 5 days, 7 days, 10 days, and 15 days at the inoculation site (~1 cm in length). Total protein was isolated and separated using SDS-PAGE. Then, a WB analysis was performed. The Xoo signal in both interactions increased proportionately with the extended inoculation time. To obtain insightful results, total proteins isolated from TP309-P6 and 4021-P6 interactions at 1, 2, 3, 4, and 5 dpi were analyzed using WB in parallel. The abundance of HSP protein in rice was used as a loading control. As shown in Figure 5, the Xoo signal could be detected in rice at 1 dpi, and significant differences in the number of Xoo cells in the resistant/susceptible reactions were found at 2 dpi. With an extended inoculation time, the difference between the two interactions became more obvious.

The distribution of Xoo in rice plants

Leaf samples, including the upper (1-6 cm), middle (6-12 cm), and lower (12-18 cm) parts of inoculated leaves and uninoculated leaves of

4021 and TP309, at 0 h, 3 days, 5 days, and 10 days after inoculation were collected, and total protein was isolated and assayed by WB using the anti-*Xoo* antibody. At 3 and 5 dpi for both samples, *Xoo* could be detected only at the inoculation site (data not shown). At 10 dpi, in the incompatible interaction (4021-P6), *Xoo* was detected only in the upper part of inoculated leaves (Figure 6). However, in the compatible interaction (TP309-P6), the *Xoo* signal was detected in the upper and middle parts of inoculated leaves. *Xoo* was not detectable in the lower parts of inoculated leaves or uninoculated leaves of both compatible and incompatible interactions. Based on the results, we concluded that, within 10 days of clipping inoculation, rice plants with or without resistance genes limited the multiplication of *Xoo* within 6 or 12 cm of the inoculation site, respectively. The quantitative difference leads to a qualitative difference.

Discussion

In this study, an immunological method was established that could detect *Xoo* conveniently *in planta*. This method provides an alternative approach to determine the quantity, propagation rate, and distribution of *Xoo* in rice, and it is expected to replace the traditional leaf-clipping method, at least in some instances.

The sensitivity of the established WB procedure is quite high, detecting as few as 3,500 *Xoo* cells, which is sensitive enough to detect *Xoo* cells at 1 dpi. Moreover, the antibody is highly specific to *Xoo*, and it is conserved among different races of *Xoo*. It did not cross-react with protein samples isolated from rice, *E. coli*, and *M. oryzae*. The WB procedure is well established in molecular biology laboratories and, in general, the vitality of *Xoo* has little influence on WB detection. Using the established procedure, resistant or susceptible interactions can be distinguished by 2 dpi, much earlier than with phenotypic observations, which usually take 5-6 days to express symptoms.



Figure 1: Titer evaluation of anti-Xoo antibodies using ELISA. ELISA plates were coated overnight with 0.2 µg of Xoo protein at 4°C. After blocking at 37°C with phosphate buffer containing 1% skimmed milk (w/v) for 2 h, plates were washed with phosphate buffer. Test sera were serally diluted with PBS, then added in duplicate to the plates and incubated for 1 h at 37°C. PBS buffer was used as the blank control and serum collected before immunization was used as the negative control (NS). After washing three times with the phosphate buffer, bound antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit antibodies, and revealed using 3'3'5'5'-tetramethylbenidine dihydrochloride and H_2O_2 in phosphate-citrate buffer. The color development was stopped by adding H_2SO_4 . The optical density was recorded at 450 nm using a spectrophotometric microplate reader.

Ab-s) Anti-Xoo antibody generated using protein isolated from sonicated Xoo cells as the immunogen. Ab-un: Anti-Xoo antibody generated using protein isolated from intact (un-sonicated) Xoo cells as the immunogen. PBS: PBS buffer; NS: negative sera.

Page 4 of 6



Figure 2: Anti-Xoo antibody specificity evaluation using a WB analysis. Total proteins were isolated from uninfected rice (TP309), *E. coli* (DH5 α), Xoo (P2, P6, P8, P9, and P10), and *M. oryzae* (TH12). SDS-PAGE separated proteins were transferred to PVDF membranes, assayed using (A) the anti-Xoo antibody, and (B) a parallel gel was stained with Coomassie blue.



Figure 3: Detection of the *Xoo* protein when mixed with rice samples using a WB analysis.

A) Total Xoo proteins were mixed with samples isolated from rice TP309 and separated by SDS-PAGE. A WB analysis was performed using the anti-Xoo antibody. HSP: The abundance of HSP protein in rice was determined and used as a loading control.

B) Quantification of signal intensity collected from three repeats of WB analysis. The average and standard derivations were calculated. The standard curve was generated and a linear regression was performed.

Additionally, compared with DNA- and RNA-based technologies, the false-positive rate is much lower using WB detection. It can also be standardized for high throughput using an automatic WB analyzer and standard reference *Xoo* samples.

Xoo infects rice leaves through hydathodes or wound sites and then enters the xylem vessels where it can multiply and spread. Smear inoculations were used to infect rice leaves and track the multiplication of GFP-tagged *Xoo*. It was found that the fluorescence intensity in the resistant rice leaves was significantly weaker than that in susceptible rice leaves at 9 dpi [27]. However, smear inoculation infected the whole rice leaf simultaneously, making it impossible to monitor the speed of *Xoo* spread *in planta*. Using PCR technology, *Xoo* was found within 6 cm of the inoculation site 2–3 days after clipping inoculation in susceptible rice-*Xoo* combinations [13, 14].

In this study, the spread of Xoo by clipping inoculation was

investigated using immunological methods. The results showed that at 10 dpi, the distance *Xoo* spread was limited to 6 cm of the inoculation site on leaves in incompatible rice-*Xoo* interactions, while in compatible interactions, the distance of *Xoo* spread was 12 cm from the inoculation site. The number of *Xoo* cells and the spread distance in resistant and susceptible combinations differed from each other. Currently, the detection of *Xoo in planta* can be achieved using either PCR or immunological methods; however, the determination of resistance or susceptibility still requires a more sophisticated analysis. Rice can tolerate certain amounts of *Xoo*, therefore, the difference between resistance and susceptibility is related to several factors, such as the presence or absence of certain *Xoo* races, the quantity of *Xoo* cells, and the presence or absence of rice resistance genes.

The establishment of this immunological method provides an effective way to investigate the plant-microbe rice-*Xoo* model system. It will be helpful in studying plant disease resistance mechanisms, and it may be used in the management of rice production. Moreover, the



Figure 4: A phenotypic analysis of rice leaves inoculated with Xoo

A. Picture of rice leaves taken at 15 days after *Xoo* inoculation. B. Average of lesion lengths on rice leaves. At least five inoculated leaves were measured, and averages and standard derivations were calculated. TP309-P6: TP309 plants inoculated with *Xoo* P6 showing a compatible interaction; 4021-P6: 4021 plants inoculated with *Xoo* P6 showing an incompatible interaction.



Figure 5: Comparison of Xoo levels in rice leaves between compatible and incompatible interactions

A) WB detection of *Xoo* levels in rice leaves at different time points after inoculation. At 1, 2, 3, 4, and 5 days after *Xoo* inoculation total proteins were isolated from rice leaves. SDS-PAGE separated total proteins were transferred to PVDF membrane and incubated with an anti-*Xoo* antibody. HSP: The abundance of HSP protein in rice was used as a loading control.

B) Quantification of *Xoo* levels detected using WB. The signal intensities were collected from three independent WBs and normalized based on the total signal intensities within a gel. The averages and standard derivations were calculated. R: Samples collected from *Xoo*-inoculated 4021 rice plants; S: Samples collected from *Xoo*-inoculated TP309 rice plants.



Figure 6: The distribution of *Xoo* in rice leaves. Samples were collected from rice leaves at 10 dpi with *Xoo*, total proteins were isolated and assayed using WB with the anti-*Xoo* antibody. 4021-P6: Samples collected from *Xoo*-inoculated 4021 plants; TP309-P6: Samples collected from *Xoo*-inoculated 209 rice plants. Up, Mid, and Low: The upper (1-6 cm), middle (6-12 cm), and lower (12-18 cm) parts of inoculated leaves, respectively; Non: Inoculated leaves from the same rice plants. HSP: The abundance of HSP protein in rice was used as a loading control.

availability of a highly specific antibody will be useful in investigating the subcellular localization of *Xoo* via immunohistochemistry and to the development of a testing strip or ELISA kit, which could be used during rapid quarantine seed inspections and even in the surveillance for rice plants in the paddy fields.

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Conflict of Interest

There is no conflict of interest between authors for this manuscript.

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Page 6 of 6

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