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ORIGINAL RESEARCH

Quantitative Measurements of *Xanthomonas Oryzae* pv. *Oryzae* Distribution in Rice Using Fluorescent-Labeling

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Abstract The rice host sensor, XA21, confers robust resistance to most strains of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the casual agent of bacterial blight disease. Using in planta fluorescence imaging of *Xoo* strain PXO99Az expressing a green fluorescent protein (*Xoo-gfp*) we show that XA21 restricts *Xoo* spread at the point of infection. This noninvasive and quantitative method to measure spatial distribution of *Xoo* populations *in planta* facilitates detailed assessment of plant disease resistance.

Keywords *Xanthomonas oryzae* pv. *oryzae*, GFP, *Oryza sativa* · *Xa21*

Abbreviations

Xoo	*Xanthomonas oryzae* pv. *oryzae*
GFP	green fluorescent protein
CFU	colony-forming unit

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Introduction

Xanthomonas oryzae pv. *oryzae* (*Xoo*), the causal agent of bacterial blight disease, causes significant yield losses worldwide (Cheatham et al. 2009). *Xoo* infects rice leaves through natural openings such as hydathodes and wounded sites (Shekhawat and Srivastava 1972; White and Yang 2009). It then enters the xylem vessels, where it can multiply and spread. Upon recognition of the sulfated peptide, $AxY^{S}22$ derived from the N terminus of the Ax21 (activator of XA21-mediated immunity) protein, the host sensor XA21 triggers robust resistance to *Xoo* (Lee et al. 2009). To quantify disease resistance, a clipping inoculation method is commonly used (White and Yang 2009). This method has facilitated the identification of numerous genes controlling plant immunity, including *Xa21*, XA21 binding protein 3 (*Xb3*), *Xb15*, and *Xb24* (Wang et al. 2006; Park et al. 2008; Lee et al. 2009; Chen et al. 2010), and others (Park et al. 2010b). A drawback to this method, however, is that it forces *Xoo* directly into the xylem vessels. Thus, this approach is not useful for studying early host responses to *Xoo* under natural infection conditions.

Quantification of bacterial populations is another important method for quantifying disease (Song et al. 1995; Park et al. 2008). However, traditional methods for counting bacterial populations disrupt leaf tissues. Furthermore, such methods are not useful for observation of the distribution of *Xoo* throughout the entire leaf. As an alternative, we and others have turned to the use of pathogens expressing fluorescent proteins (Valdivia et al. 1996; Lagopodi et al. 2002; Ju et al. 2005; Venard and Vaillancourt 2007). For example, we recently developed a fluorescent *Xoo* strain expressing the green fluorescent protein (*Xoo-gfp*) to study the distribution of *Xoo* cells *in planta* and to quantify bacterial multiplication (Han et al. 2008).

Despite these advances, it is still unknown how *Xoo* cells penetrate and spread under natural infection condition or how XA21-mediated immunity restricts *Xoo* propagation and invasion into leaves. To address these questions, here we report the use of a smear inoculation method that more closely mimics natural infection conditions (Shekhawat and Srivastava 1972). In combination with noninvasive fluorescence imaging of *Xoo-gfp*-inoculated leaves, we have identified points of bacterial entry and monitored bacterial distribution.

Materials and Methods

Plant and Bacterial Materials

Seeds of Kitaake, a rice (*Oryza sativa* L.) cultivar, and a transgenic Kitaake plant transformed with *myc-Xa21* driven by the *ubiquitin* promoter (Ubi Myc-*Xa21* (Park et al. 2010a), abbreviated as XA21-Kitaake throughout text) were germinated by imbibing in water for 7 days and then planted in clay soil with slow fertilizer (APEX 14–14–14/14–6–11.6 Elemental (Metric); Simplot, USA). Chelated iron (0.6 g l^{-1} Sprint 330, Becker Underwood, USA) was applied to water every week. Plants were maintained in a growth chamber under 16 h light:8 h dark conditions with 28°C. Healthy and well-expanded leaves from 4- to 5-week-old rice plants were used for inoculation.

Xoo PXO99Az (carrying Ax21 activity) (Song et al. 1995; Lee et al. 2009) expressing GFP (*Xoo-gfp*) (Han et al. 2008) was grown on PSA plate (Peptone Sucrose Agar, 10 g l^{-1} peptone, 10 g l^{-1} sucrose, 1 g l^{-1} glutamic acid, 16 g l^{-1} agar, and pH 7.0) containing carbenicillin (100 μg l^{-1}) for 3 days and suspended with water at OD=0.5 (600 nm) for inoculation. Three layers of gauze were soaked with *Xoo* suspension and smeared onto leaves three times. For mock treatment, water was used instead of the *Xoo* suspension.

Detached Leaf Assay

Leaves from rice plants were cut and the basal regions trimmed so that the detached leaves were ca. 10 cm long. The detached leaves were kept moist in 50-ml plastic tubes. The detached leaves were surface sterilized with 70% EtOH for 1 min and rinsed with sterile water twice. Because previous studies have demonstrated that leaf discs floated overnight do not show symptoms of wounding (Heese et al. 2005, 2007; Lu et al. 2009), the sterile detached leaves were maintained in a growth chamber under 16 h light:8 h dark conditions with 28°C for 1 day to recover from wounding. The detached leaves were rubbed with sterile gauze and then dipped into *Xoo* suspension for 3 s. The excess *Xoo* suspension was drained and the inoculated detached leaves kept in 15-ml sterile test tubes with 3 ml sterile water under light/dark cycles (16 h light:8 h dark). Four days after inoculation, we measured fluorescence from the detached leaves.

Fluorescence Imaging and Microscopic Observation of *Xoo-gfp*

The fluorescence imaging mode of NightOWL II LB983 with an NC100 camera (Berthold Technologies, USA) was used for *Xoo-gfp* imaging using the associated software (IndiGo). Throughout our experiments, we used the same exposure times and light conditions. We repeated imaging twice. Representative images are presented in each figure. Microscopic observation of *Xoo-gfp* was done using a Zeiss Axiophot fluorescence microscope (Jena, Germany). Adaxial sides of leaves were observed using a GFP filter set (excitation, 470/40 nm; beam splitter, FT495 nm, emission, 525/50 nm) at ×100 magnification as described previously (Han et al. 2008).

Xoo Population Measurements

Known concentrations (colony-forming unit (CFU) per ml) of *Xoo-gfp* cell suspensions (200 μl) were added to wells of a 96-well plate and the fluorescence from each well was measured by IndiGo to examine the correlation between *Xoo-gfp* number and *Xoo-gfp* fluorescence levels (Fig. 2a). NIH ImageJ software (http://rsb.info.nih.gov/ij) was used to quantify fluorescence levels of *Xoo-gfp* (integrated gray value, IntDen), and leaf areas (mm^2). Fluorescence per leaf area was obtained by dividing the fluorescence level by leaf area (mm^2). To compare *Xoo-gfp* fluorescence per leaf area with established methods for assessing *Xoo* populations, populations of *Xoo-gfp* cells in leaves were assessed using the cell-counting method (Kauffman et al. 1973; Song et al. 1995; Lee et al. 2009). The resultant *Xoo* CFU was divided by leaf area (mm^2) to obtain CFU per leaf area. To evaluate the correlation between *Xoo-gfp* number and *Xoo-gfp* fluorescence level, linear regression analysis between fluorescence level per leaf area and CFU per leaf area were calculated using a the statistic software environment package, R (http://www.R-project.org/)(R Development Core Team 2005). Results from this analysis were used to convert fluorescence level per leaf area into CFU per leaf are for Figs. 3 and 5, and Figs. S1 and S3. “Fire LUT” in ImageJ was used for pseudocoloring of *Xoo-gfp* distribution *in planta* in Fig. 3, and Figs. S1 and S3. Minimum and maximum pixel values were fixed (924 and 12,000) for pseudocoloring based on Fig. 2b.

Results and Discussions

Validation of Fluorescence Imaging of *Xoo-gfp in Planta*

To validate in planta fluorescence imaging, we inoculated rice plants with *Xoo-gfp* using the clipping method. Mock-treated leaves did not show any significant fluorescence, whereas a *Xoo-gfp* inoculated Kitaake leaf, susceptible to *Xoo* strain PXO99Az, displayed strong fluorescence (Fig. 1a). The limited distribution of *Xoo-gfp* fluorescence at the clipped site of the XA21-Kitaake leaf (Park et al. 2008) indicates that *Xoo-gfp* cells are unable to spread. As observed previously (Han et al. 2008), the *Xoo-gfp* distribution is consistent with measurements of the lengths of water-soaked lesion, typical of infected leaves (Fig. 1b). These results confirm that in planta fluorescence imaging can be used to quantify *Xoo-gfp* infection.

Quantification of Disease Resistance through Fluorescence Imaging

We next examined if levels of fluorescence reflect the numbers of *Xoo* cells. Figure 2a shows that fluorescence positively correlates with *Xoo* cell numbers between 2.0×10^4 and 1.0×10^7 CFU/leaf area (mm^2) as shown by linear regression analysis (R^2=0.9912, $p<1\times10^{-15}$ with *F*-test). To test if this correlation holds true *in planta* as well, we examined fluorescence levels from in leaves infected with *Xoo-gfp* and assessed bacterial populations by established cell counting methods (Kauffman et al. 1973). We observed a clear correlation between the two types of measurements shown by linear regression analysis (R^2= 0.7467, $p<0.0002$ with *F*-test; Fig. 2b). The stronger correlation in vitro (Fig. 2a) than *in planta* (Fig. 2b) is likely due to reduced handling before imaging *Xoo-gfp* fluorescence (see Materials and Methods). These results indicate that *in planta* fluorescence imaging of *Xoo-gfp* is a powerful method for quantifying *Xoo* populations.

In Planta Distribution of *Xoo* Cells after Smear Inoculation

Under natural field conditions, rice plants are wounded by wind, wind-blown rain, and hail leaving them exposed to *Xoo* infections (Nino-Liu et al. 2006). To mimic these natural field conditions, the smear inoculation method is quite useful because it induces mild surface wounds without exposing the xylem to infection. We therefore used our fluorescence imaging method to visualize *Xoo* distribution on plants inoculated using the smear method. Immediately after inoculation, no significant fluorescence can be observed in leaves of either Kitaake or XA21-Kitaake (Fig. S1). This result is consistent with the low *Xoo* cell population (2.2×10^3 CFU in Kitaake and 2.3×10^3 CFU in XA21-Kitaake) measured by the cell counting method. Nine days after inoculation, we observed stronger fluorescence in Kitaake than XA21-Kitaake (Fig. 3), suggesting that *Xoo-gfp* cells preferentially propagate in susceptible Kitaake leaves. In Kitaake leaves we observed *Xoo* cells spreading along veins, which is consistent with the known

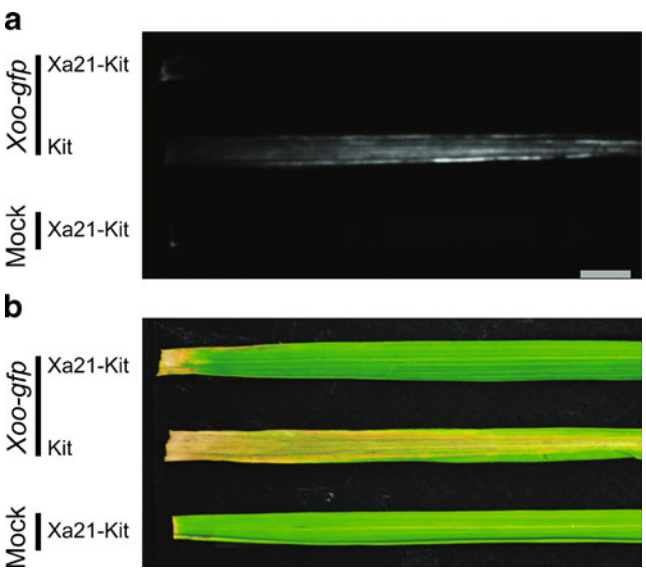

Fig. 1 *Xoo-gfp* specific signals of infected rice leaves correspond to water-soaked lesions. **a** Fluorescence image and **b** bright image were obtained from identical leaves of the susceptible cultivar (*Kit* Kitaake) and the resistant line (*Xa21-Kit* XA21-Kitaake) with *Xoo-gfp* or mock inoculation

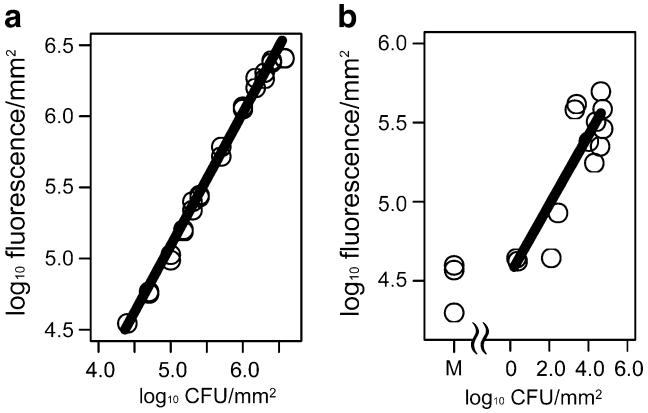

Fig. 2 Quantifications of *Xoo-gfp* populations by fluorescence imaging. **a** Correlation between *Xoo-gfp* number and *Xoo-gfp* fluorescence level. Known concentrations of *Xoo-gfp* cell suspensions (200 μl) were added to wells of a 96-well plate and the fluorescence from each well was measured. Duplicate samples for each well were measured. **b** *In planta* correlation between *Xoo-gfp* number and *Xoo-gfp* fluorescence. Leaves were harvested 0 and 9 days after inoculation with *Xoo-gfp*, and the fluorescence of inoculated leaves was measured. The numbers of *Xoo* cells in the leaves were counted using our established assay method (Kauffman et al. 1973; Song et al. 1995; Lee et al. 2009). *M* mock treated

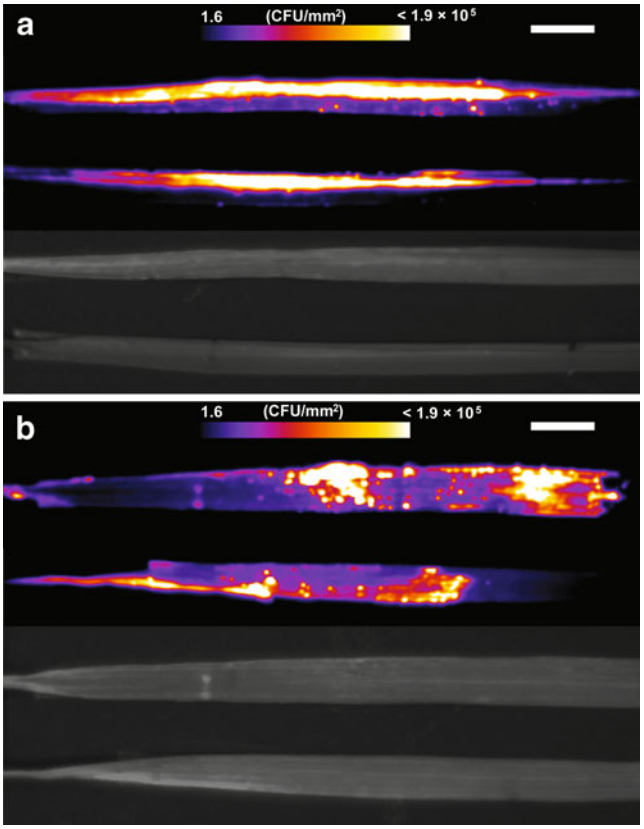

Fig. 3 Differential distribution of *Xoo-gfp* in rice leaves of **a** susceptible Kitaake and **b** resistant XA21-Kitaake. *Upper* and *lower panels* indicate fluorescence and bright field images, respectively. *Bar*, 1 cm. A *color bar* indicates *Xoo-gfp* concentration (CFU/mm^2) based on results in Fig. 2b. Images were taken 9 days after inoculation with *Xoo-gfp* (OD=0.5 at 600 nm)

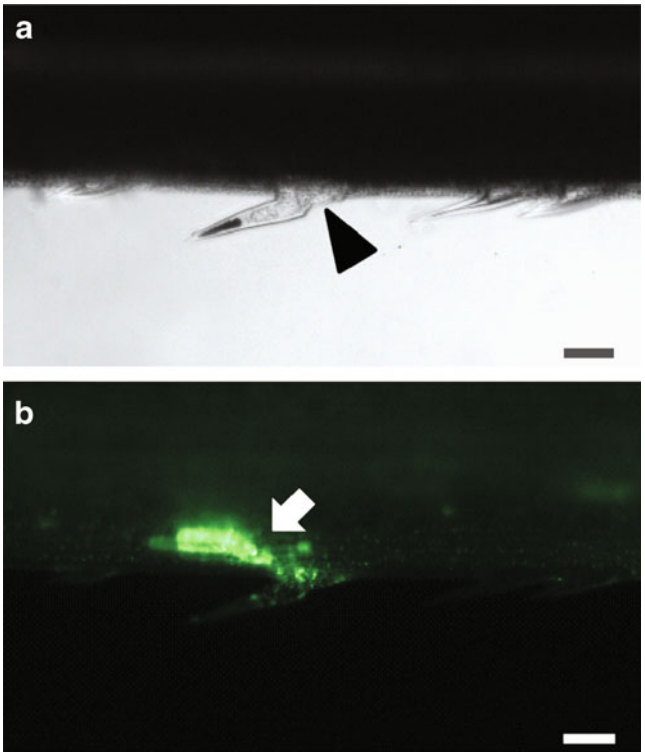

Fig. 4 Microscopic observation of *Xoo-gfp* distribution at the point of entry 4 days after smear inoculation. **a** *Xoo-gfp* cells (indicated by an *arrow*) on a XA21-Kitaake leaf were visualized by using GFP filter set (excitation, 470/40 nm; beam splitter, FT495 nm, emission, 525/50 nm) at ×100 magnification. **b** An identical image was obtained under a bright field using a Zeiss Axiophot fluorescence microscope (Jena, Germany) at ×100 magnification and overlaid with **a**. *Xoo-gfp* cells were observed below the broken trichome (indicated an *arrowhead*), which is presumably at the point of entry of the bacterial cells. *Bars* (for **a** and **b**), 50 μm

multiplication of *Xoo* in xylem vessels (Nino-Liu et al. 2006; Han et al. 2008). In contrast, the XA21-Kitaake restricted *Xoo-gfp* spread as reflected by the limited fluorescence observed. In the leaves, a high density of *Xoo* cells is observed only in a localized area. To assess the type of cells found in the sites, we carried out microscopic observations (100×). These studies revealed that high densities of *Xoo-gfp* cells are mainly localized at broken trichomes in XA21-Kitaake leaves (Fig. 4; Fig. S2a). In contrast, in Kitaake leaves, *Xoo* cells spreading beyond the site of infection at broken trichomes into the xylem vessels, as reflected by strong fluorescence signals in the xylem (Fig. S2b). Broken trichomes are well-known site of entry for the pathogens *Pseudomonas tomato* (Bashan et al. 1981) and tobacco mosaic virus (Huang 1986). We rarely observed high densities of *Xoo-gfp* cells around hydathodes under the microscope. This result suggests that we can obtain a higher efficiency inoculation through trichomes than through intact hydathodes. Taken together, our results indicate that *Xoo* penetrates into leaves through broken trichomes, a condition commonly induced by winds, wind-blown rain, and hail.

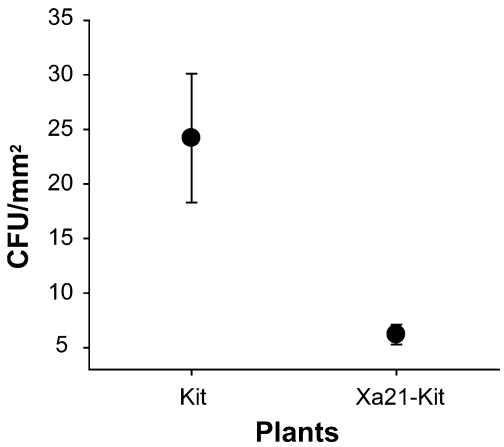

Fig. 5 Xa21-Kitaake resistance to *Xoo* revealed by quantification of in planta fluorescence images of detached leaves. Error bars show SEM of two independent experiments is shown; n=16 detached leaves. *Kit* Kitaake, *Xa21-Kit* XA21-Kitaake

Development of Quantitative Assay System to Measure Resistance to *Xoo*

To facilitate a rapid and quantitative assay system, we applied our smearing inoculation method to detached rice leaves. For this "rapid inoculation," we first rubbed detached leaves with sterile gauze and then dipped the leaves into *Xoo* suspension for 3 s. Excess *Xoo* suspension was drained and the inoculated detached leaves were kept in sterile water under light/dark cycles (16 h light:8 h dark). Four days after inoculation, we measured fluorescence from detached leaves of either Kitaake or XA21-Kitaake plants. Resistance to *Xoo* could easily be observed in XA21-Kitaake compared with Kitaake leaves (Fig. 5; $p<0.01$ with Student's *t*-test). This result shows that, in combination with the smear inoculation method, the fluorescent assay is rapid and useful to quantify resistance to pathogens.

In Planta Fluorescence Imaging of *Xoo* Reveals Distribution in Stem and Sheath Tissues

The clipping inoculation method can be applied only to leaf blades because clipping of other tissue types yields high levels of damage, which makes it difficult to measure infection. We therefore tested if the smear inoculation method combined with in planta fluorescence imaging can facilitate visualization of *Xoo* distribution in stem and sheath tissues. There was no obvious fluorescence in the absence of *Xoo-gfp* (Fig. S3, bottom). After smear inoculation of stem and sheath tissue (approximately 5 cm length, a bracket in Fig. S3) in Kitaake, strong *Xoo-gfp* fluorescence could be observed throughout the entire tissue (Fig. S3, top), suggesting that *Xoo* cells spread out from the point of inoculation. In contrast, weaker *Xoo-gfp* fluorescence within the inoculated area was observed in XA21-Kitaake plants (Fig. S3, middle). As shown in Fig 2b, the fluorescence reflects 5.0×10^4 (CFU/mm^2) in Kitaake and 3.8×10 (CFU/mm^2) in XA21-Kitaake. These results demonstrate that (1) *Xoo-gfp* distribution is detectable in stem and sheath cells and (2) XA21-mediated immunity is present in stem and sheath not only in leaf blades. Therefore, our method of inoculation and detection of *Xoo* is potentially useful for studies of host disease resistance in many types of plant tissues.

Conclusion

Here we show that *in planta* fluorescence imaging of *Xoo-gfp* in rice leaves using the smear inoculation method is useful for detecting distribution of *Xoo* cells inside intact leaves in a quantitative manner. This imaging method delivers more detailed phenotype information on the mechanism of plant disease resistance. For example, these studies reveal that XA21-mediated immunity restricts *Xoo* spread at the point of entry of broken trichomes, inhibiting-propagation to rice xylem vessels. The smear method was also used to demonstrate XA21-mediated immunity in the stem and sheath, not previously revealed using conventional inoculation methods.

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Authorship P.C.R., C.J.P., and K.N. contributed to project design and performed all experiments except microscopic observation (done by C.J.P). Image analysis was done by K.N., whereas P.C.R., C.J.P. and K.N. wrote the paper. All authors discussed the results and commented on the manuscript.

Conflict of interest The authors declare no competing financial interests.

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