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Influence of rice development on the function of bacterial blight resistance genes

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Abstract Disease resistance genes most commonly used in breeding programs are single, dominant genes with relative effectiveness that is sometimes influenced by plant developmental stage. Knowing the developmental stages at which a resistance gene is functional is important for disease management. In rice, resistance at the seedling stage is crucial, because wounding during transplanting increases the potential for bacterial blight disease, and not all bacterial blight resistance genes are effective at the seedling stage. Effectiveness of the bacterial blight resistance genes *Xa4, xa5*, and *Xa7,* all in a common genetic background, was evaluated at different developmental stages by measuring lesion length and bacterial numbers after inoculation with the bacterial pathogen, *Xanthomonas oryzae* pv. *oryzae*. The *Xa4* and *xa5* genes controlled disease at all growth stages. In contrast, *Xa7* was not fully functional in very young seedlings, but was completely effective by 21 days after sowing (das). The effects of plant developmental stage on interactions of the *Xa7* gene with *X. oryzae* pv. *oryzae* strains carrying different mutant *avrXa7* alleles were also tested. If a partial or fully functional *avrXa7* allele was present, *Xa7* resistance was effective at all growth stages tested after the transplant stage (>21 das).

Keywords Aggressiveness · Avirulence/effector gene · *Oryza sativa* · Virulence · *Xanthomonas oryzae* pv. *oryzae*

Abbreviations

Avr	avirulence gene
BB	bacterial blight
Dai	days after inoculation
Das	days after sowing
R gene	resistance gene
Xoo	*Xanthomonas oryzae* pv. *oryzae*

Introduction

Understanding factors that influence function of disease resistance genes and their regulation is important for management of plant disease. Disease resistance genes most commonly used in breeding programs are single, dominant resistance (*R*) genes that either directly or indirectly recognize products of

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pathogen effector (*avr*) genes (Flor 1971; Keen 1990). These interactions, which are referred to as gene for gene interactions, initiate a signal transduction pathway that often, but not always, leads to a rapid and localized plant cell death known as the hypersensitive response (HR) (Goodman and Novacky 1994; Greenberg 1997). If the pathogen lacks the effector (*avr*) gene or the host lacks the corresponding plant *R* gene, disease susceptibility results. The effectiveness of *R*-gene mediated resistance in restricting bacterial colonization and host phenotypic responses, including the timing and strength of the HR, varies considerably depending on the specific *R* gene involved, the mode of interaction/recognition of the specific *R* gene (direct or indirect), as well as contributing growth factors such as environmental conditions (reviewed in Rafiqi et al. 2009). For example, Tao et al. (2003) used gene expression profiling to show that while shape and amplitude of expression profiles were similar in two different *R* gene mediated incompatible reactions in Arabidopsis, the *RPS2* mediated responses were generally slower and less strong than the *RPM1* mediated responses, and this was illustrated by a slower onset of the hypersensitive response.

Variation in effectiveness of *R* genes is particularly evident when assessed at different stages of plant development. For example, in wheat, cultivars Thatcher and Red Bobs are both susceptible to race 9 of the leaf rust pathogen (*Puccinia recondita)* at the seedling stage. By plant maturity, however, Thatcher becomes resistant to race 9, whereas Red Bobs remains susceptible (Bartos et al. 1969). Some resistance genes are functional in seedlings, including the seeds themselves, as well as adult plants. In lettuce, two recessive resistance genes $mo1^1$ and $mo1^2$ are effective in seeds and confer resistance to seedlings to *Lettuce Mosaic Virus (*LMV) (German-Retana et al. 2008).

Determining the effect of plant developmental stage on disease resistance is complicated by the fact that plants are often more resistant to disease as they mature, a phenomenon known as "adult plant resistance" (Van der Plank 1963; Van der Plank 1982). Van der Plank (1963) postulated that adult plant resistance is generally quantitative (or horizontal) resistance, as it is often polygenic, considered to be non-race specific, and results in resistance that is usually not complete, i.e. smaller pustules, slower hyphal growth, or slower infection rates. For example, in oats, less crown rust develops on adult plants than on seedling plants (Heagle and Moore 1970). Although more moderate as compared to resistance conferred by single dominant resistance genes, this type of adult plant resistance did increase with plant age (Heagle and Moore 1970).

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. o*ryzae (Xoo)* imposes significant disease pressure on cultivated rice (*Oryza sativa* L.), particularly in irrigated and rainfed rice growing regions of Asia (Mew et al. 2004). Risk for BB infection is high in seedling plants, which are wounded and stressed during transplanting. Most sources of resistance to BB used in breeding programs are single dominant *R* genes (Mew 1987). The effectiveness of resistance at different developmental stages has been previously tested for many of these genes (Cao et al. 2007a; b; Mazzola et al. 1994; Sidhu and Khush 1978; Zhang and Mew 1985). Mazzola et al. (1994) demonstrated that effectiveness of one *R* gene, *Xa21,* was developmentally regulated by using two measures of disease development, lesion length and bacterial colonization of leaves. If seedling plants were inoculated at less than 21 days after sowing (das), no differences in disease development were observed between the susceptible cultivar (IR24) and the near-isogenic cultivar containing the *Xa21* gene. As plants matured from 21 to 51 das, however, disease was lower than in IR24, indicating *Xa21*-mediated resistance was effective. Century et al. (1999) found that *Xa21* resistance progressively increases from a susceptible interaction at the second leaf stage, as it matures to the adult leaf stage (9/10 leaf stage). Interestingly they found that *Xa21* expression was independent of the plant developmental stage and that the *Xa21* gene transcript is not correlated with expression of *Xa21.* Therefore they postulated that the developmental regulation of *Xa21* disease resistance is controlled either post-transcriptionally, or by other factors (Century et al. 1999). *Xa6* also does not function at the seedling stage (younger than the 4 to 5 leaf stage), however, it does confer resistance in older plants (older than 7 to 8 leaf stage) (Sidhu and Khush 1978). Another BB *R* gene, *Xa10,* is functional at the seedling stage (less than 21 das) while in contrast, *Xa4* function is not clearly identifiable until maximum tillering (Sidhu and Khush 1978; Zhang and Mew 1985). Anecdotal reports suggested that young plants (30 days after sowing) of rice cultivars carrying the *Xa7* gene were less resistant than adult plants (C. Vera Cruz, pers. communication). The stage of plant development at which *R* genes are effective also varies

among the host backgrounds in question. Effectiveness of *Xa3*, which recently was shown to be the same as the *Xa26* gene (Xiang et al. 2006) depends on host genetic background. In this case, different genetic backgrounds showed effectiveness at either or both seedling and adult stages and resistance was higher in *japonica* as compared to *indica* backgrounds (Cao et al. 2007a; b).

Understanding the range of developmental phases in which a rice BB *R* gene is effective is particularly important for the deployment of those genes, especially in rice where seedlings are often wounded during transplanting. Because the pathogen gains access to the vascular system through wounds, seedlings are highly vulnerable to infection during transplanting (reviewed in Mew 1987). We investigated the impact of plant development stage on the interactions of rice containing the *R* genes *Xa4, xa5*, and *Xa7* with *Xoo* strains containing the corresponding *avr* effector genes. These three *R* genes were selected because the developmental stage at which they function is either unknown or remains controversial. Knowing the growth stages at which these genes are effective may inform how they could be used in breeding and disease management programs. Direct comparisons of the functions of the different *R* genes were facilitated by the availability of near-isogenic lines containing each gene independently (Ogawa et al. 1991), as well as bacterial strains that contain no, one, or all three *avr* effector genes that correspond to *Xa4, xa5* and *Xa7*, respectively.

Materials and methods

Growth of bacteria and rice cultivars

For evaluation of the impact of plant development stage on expression of resistance, the *Xoo* strains used were PXO99 (compatible on all genotypes, no relevant *avr* alleles) and PXO145 (*avrXa4*$^+$, *avrxa5*$^+$, and *avrXa7*$^+$). In experiments involving additional analysis of *xa5* gene interactions, strain PXO86 (*avrxa5*$^+$) was used. *Xoo* inoculum was prepared to a bacterial density of 5×10^8 CFU as described previously (Hopkins et al. 1992).

Rice lines included the recurrent parental rice line IR24 that contained no relevant *R* genes and is susceptible to all *Xoo* strains used, and the near-isogenic lines, IRBB5 (*xa5),* IRBB4 (*Xa4),* and IRBB7 (*Xa7*) (Ogawa et al. 1991). All plantings were done in a split-split plot design with growth stage applied as the whole plot factor and cultivar as the split plot factor.

Plant inoculation, disease evaluation, and assessment of bacterial numbers

Plants were inoculated at 11 and 21 das (seedling stage), 31 and 41 das (maximum tillering), 51 and 71 das (booting stage) using a leaf-clip method (Mazzola et al. 1994). One to ten of the youngest, fully expanded leaves were inoculated on each plant. At 14 days after inoculation (dai), we assessed two measures of disease development in inoculated leaves, i.e., lesion length (cm) and the bacterium's ability to colonize the plant (log CFU per leaf). To assess bacterial numbers, leaf homogenates were serially diluted (up to 10^{-6}) with sterile ddH_2O and plated onto Suwa's media (Suwa 1962) amended with cephalexin (20 ug/ml) and cycloheximide (50 ug/ml). After incubation at 28°C for 24–48 h, bacterial colonies were counted. Bacterial numbers were not determined from plants inoculated at 11 das because lesions expanded well into the stems of the plants, and leaves were often dead. The experiment was repeated over three independent time periods. Significant differences for lesion length and bacterial numbers were compared by ANOVA using a PROC MIXED procedure in the SAS statistical program (SAS Institute, 1992, Cary, NC).

To more rigorously test the effectiveness of *xa5* at different growth stages, rice lines IRBB5 and IR24 were grown in the greenhouse to 21 (seedling stage with two fully expanded leaves), 28, 35 and 42 das (maximum tillering). *Xoo* strains PXO86 (*avrxa5*$^+$) and PXO99 (*avrxa5*$^-$) were individually inoculated to one leaf per plant and lesion length (cm) was measured at 15 dai.

Influence of *avrXa7* allele on interactions with rice containing *Xa7* at different developmental stages

Effects of plant developmental stage on interactions with the *Xa7* gene were evaluated with *Xoo* strains carrying different mutant *avrXa7* alleles. Rice cultivars IRBB7 and IR24 were inoculated at three different ages, i.e., 28, 37 and 51 das over two experiments. In

this study, due to cooler greenhouse temperatures, plants at 28 das were at the seedling stage (two fully expanded leaves) and plants at 51 das were at maximum tillering. We used *Xoo* strains for inoculation that exhibited a range of phenotypes on IRBB7 rice (Ponciano et al. 2004). These strains range from avirulent (*avrXa7*$^{+}$, PXO1865, PXO368), to partially virulent (*avrXa7* $^{-/+}$, PXO0314, PXO441, PXO357, PXO433, PXO356, PXO348, PXO448, PXO419), to virulent (*avrXa7* $^{-}$, PXO346, PXO2684). Lesion length (cm) was measured at 6, 9, 12, 15, and 18 dai. The area under the disease progress curve (AUDPC) was calculated (Campbell and Madden 1990) and significant differences were compared using proc mixed in the SAS statistical program (SAS Institute, 1992, Cary, NY).

Results

General adult plant resistance

Experiments were first conducted to to determine if *Xoo* strains used for these studies were equivalent in their ability to cause disease. This was accomplished by comparing the amounts of disease induced by each strain on the susceptible host (IR24) at different plant growth stages (Fig. 1a and b). There was no difference in the lesion lengths that PXO99 and PXO145 induced on IR24 at each growth stage tested (all p-values>0.05; data not shown). Regardless of the *Xoo* strain used for inoculation, as the plants matured lesion lengths on the susceptible variety (IR24) were generally shorter than on younger plants (>51 das, all p-values<0.05) (Fig. 1a and b). Similarly, lesion lengths on IRBB4, IRBB5 and IRBB7 using a compatible isolate (PXO99) were longer on younger plants relative to plants inoculated when older (Fig. 1a and b). This demonstrated that rice plants exhibit an adult plant resistance that is not necessarily dependent on the presence of a described *R* gene in the host.

Developmental regulation of *Xa4, xa5,* and *Xa7* measured by lesion length and bacterial accumulation

Both IRBB4 (*Xa4)* and IRBB5 (*xa5)* showed shorter lesions (resistance), as compared to IR24, at all plant growth stages when inoculated with *Xoo* strain PXO145 (all p-value's<0.05, Fig. 1b). In contrast, at 11 das, IRBB7 (*Xa7)* did not show significant levels of resistance relative to IR24 after inoculation with the incompatible strain PXO145 (p-value=0.08, Fig. 1b). This suggests that *Xa7* is not fully functional in very young seedlings, but is effective by 21 das. Plants inoculated at 11 das typically exhibited lesions that extended into the plant stem making assessment of lesion lengths difficult and contributing to increased variation in measurements. Other than IRBB7 at 11 das, all cultivars were more resistant than IR24 at all other developmental stages (Fig. 1b).

To complement the lesion length measures of disease development, bacterial numbers were measured in inoculated plants to determine *Xoo's* ability to multiply in the host. In all compatible interactions with PXO99 (Fig. 2a), bacterial numbers were high

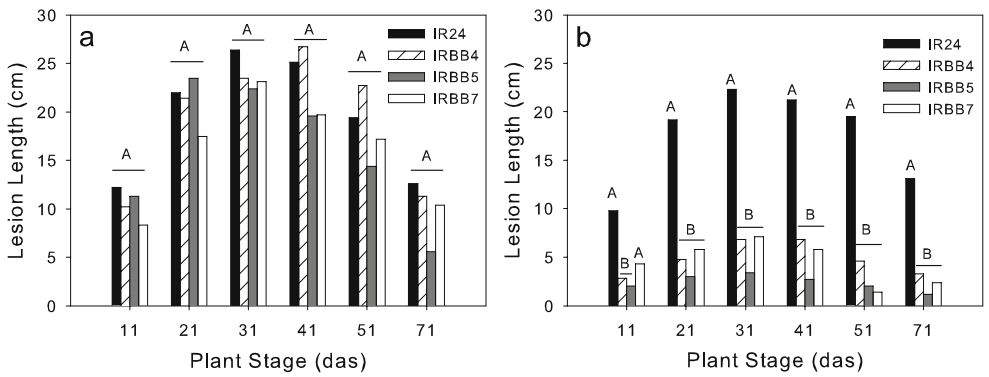

Fig. 1 **a** and **b** Lesion lengths induced by *Xoo* strains PXO99 **a** and PXO145 **b** on rice lines IR24 (susceptible), IRBB4 *(Xa4)*, IRBB5 *(xa5)*, and IRBB7 *(Xa7)* at different plant growth stages as days after sowing (*das*). Within a growth stage, capital letters indicate significant difference of lesion lengths as compared to IR24 (p-value 0.05) and vertical bars topped by the same letter are not significantly different (at p-value 0.05)

(>10^6 cfu/leaf), regardless of developmental stage. In incompatible interactions with strain PXO145, lower bacterial numbers were measured in plants containing either *xa5* or *Xa7*, regardless of plant growth stage at time of inoculation (at p-value<0.05) (Fig. 2b). Lower bacterial numbers were also observed in all comparisons for IRBB4 (*Xa4)*, except for plants inoculated at 41 das (p-value=0.54); in this case, bacterial numbers were not significantly lower when compared with the compatible interaction (Fig. 2b). Thus, although lesion length data for IRBB4 plants inoculated at 41 das (with PXO145) were restricted, suggesting resistance (Fig. 1b), bacterial numbers accumulated to levels similar to a susceptible interaction.

Because of anecdotal reports that *xa5* is not functional in younger plants, we wanted to characterize more fully this interaction using a different *Xoo* strain that harbored the *avrxa5* gene (PXO86), and which is incompatible on rice with *xa5*. In this experiment, even though PXO99 does not contain the *avrxa5* gene, it did cause shorter lesions on IRBB5 (*xa5*) than it did on the susceptible host (p-value<0.0001) at all plant stages (Fig. 3a). This indicated that, under these experimental conditions, IRBB5 may express a relatively low-level of resistance, and this resistance is not necessarily specific to PXO99. Despite this, IRBB5 again exhibited effective resistance at each growth stage tested when compared to the susceptible line (Fig. 3b). However, these results were confounded by differences in overall strain aggressiveness i.e. PXO86 was less aggressive than PXO99 (p-value<0.0001) for all developmental stages tested (Fig. 3a and b). To resolve the effects of differences in *Xoo* strain aggressiveness, we compared the ratio of lesion lengths on IRBB5 to IR24 for both strains over time (Fig. 4). The ratio of lesion lengths caused by PXO99 or PXO86 on IRBB5 relative to IR24 was approximately the same regardless of the growth stage of the plant at the time of inoculation (Fig. 4). Relative to the ratios observed after inoculation with the compatible strain (PXO99), interactions with the incompatible strain (PXO86) resulted in lower ratios and indicated that resistance was effective at each developmental stage (Fig. 4). Taken together, the results with PXO145 and PXO86 indicate that *xa5* resistance is functional at each developmental stage.

Influence of *avrXa7* allele on interactions with rice with *Xa7* at different developmental stages

Ponciano et al. (2004) demonstrated that *Xa7*-mediated resistance varied in interactions with different *avrXa7* mutant alleles. In particular, strains classified as race 9b (*avrXa7*$^{-/+}$) contained altered *avrXa7* alleles, and caused intermediate levels of disease on IR24 and low disease on IRBB7 relative to race 3 (*avrXa7*$^{+}$) and 9a/c (*avrXa7* $^{-}$) strains. Our work here suggests that *Xa7* clearly functions after 21 das. Because of the variation in interactions with the race 9b strains observed in Ponciano et al. (2004), we wanted to determine if interactions of *Xa7* and different *avrXa7* alleles are affected by developmental stage. For these experiments, we measured the area under the disease progress curve (AUDPC) (Campbell and Madden 1990) of IRBB7 and IR24 plants during interactions with race 3, 9b, and 9c strains. AUDPC provides a

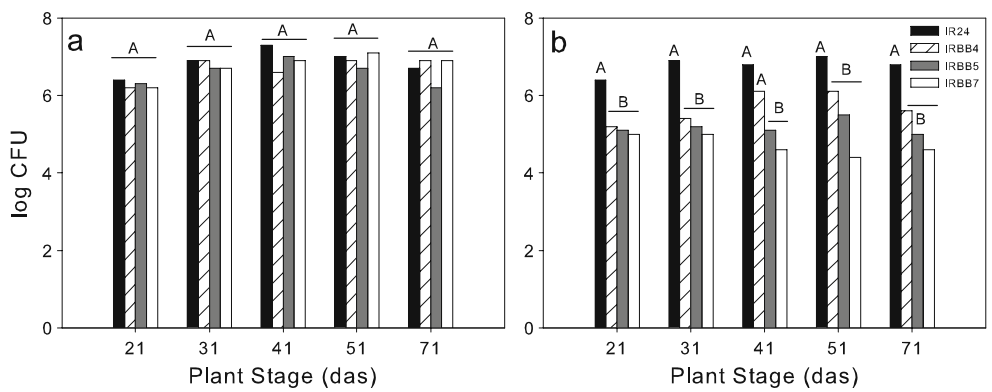

Fig. 2 **a** and **b** Bacterial numbers (log CFU) of *Xoo* strains PXO99 **a** and PXO145 **b** on rice lines IR24 (*susceptible*), IRBB4 *(Xa4)*, IRBB5 *(xa5)*, and IRBB7 *(Xa7)* at different plant growth stages as days after sowing (*das*). Within a plant growth stage, capital letters indicate significant difference of bacterial numbers of *Xoo* in a line as compared to IR24 (p-value=0.05)

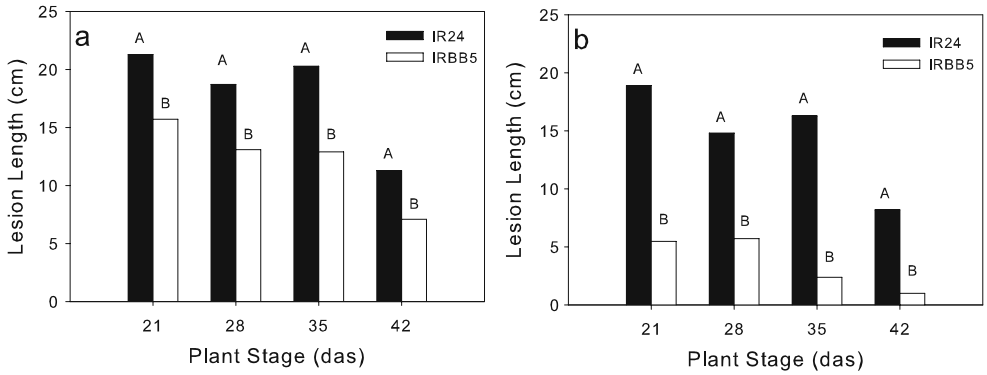

Fig. 3 **a** and **b** Lesion lengths induced by *Xoo* strains PXO99 **a** and PXO86 **b** on rice cultivars IR24 (*susceptible*) and IRBB5 *(xa5)* at different plant stages as days after sowing (*das*). Capital letters indicate significant differences between rice lines within a plant growth stage ($p<0.05$)

measure of disease progress over time; therefore, treatments with larger AUDPC have higher overall amounts of disease over time.

Considering all comparisons, the control race 9c strain, PXO2684 (*avrXa7* $^-$) had a mean AUDPC of 99.5 on IRBB7 and 131.5 on IR24, indicating that this strain was virulent to each variety (p-value=0.11). Plant stage did not affect overall disease progress on either rice line IRBB7 (*Xa7*) or IR24 (p-value=0.82). In general, disease progress over time was significantly lower on IRBB7 (*Xa7*) than IR24, confirming that IRBB7 was more resistant than IR24 for all plant stages tested with a functional *avrXa7* allele (race 3's and race 9b's) (p-value=0.0079; Fig. 5a and b). While the strain of *Xoo* inoculated did have a significant effect on disease development over time, *Xa7* resistance was effective at all plant ages tested (Fig. 5b). On IRBB7, the race 3 strains with a functional *avrXa7* (PXO1865 and PXO368) caused the least amount of disease whereas the two race 9a/c strains (PXO346 and PXO2684, *avrXa7* $^-$) caused the most disease over time (Fig. 5b). The race 9b strains caused a continuous range in AUDPC on both IR24 and IRBB7, between the two extremes, as predicted from our previous work (Fig. 5b) (Ponciano et al. 2004).

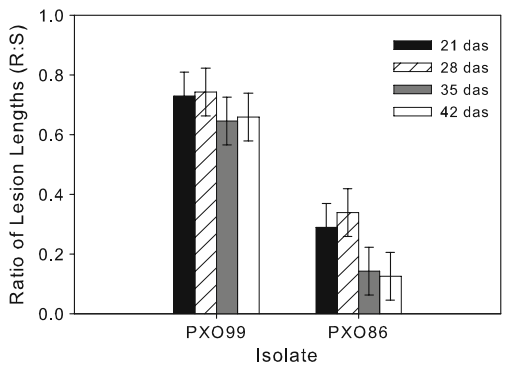

Fig. 4 Using a ratio of lesion lengths on IRBB5 (*xa5*) to IR24 (*susceptible*) plants at different days after sowing (*das*) when inoculated with *Xoo* strains PXO99 and PXO86. Error bars indicate standard error of the mean

Discussion

Using two indicators of disease development, lesion length and ability of the pathogen to multiply in the host, effects of plant growth stage on resistance conferred by three resistance genes, *Xa4, xa5,* and *Xa7,* were evaluated. After 21 das, all lesion lengths were shorter than the susceptible control, indicating that the three genes are effective in seedlings. Based on our results, deployment of *Xa4, xa5* and *Xa7* does not depend on the plant growth stage at time of transplanting (21 das). While *Xa7* did not show resistance in very young seedlings (11 das), in agronomic practice transplantation (and hence wounding) is done when plants are at least at the second fully expanded leaf (approximately 21 das). Therefore *Xa7* should be effective at a critical stage in the production cycle. During interactions with *xa5* and *Xa7,* bacterial population data supported the conclusions from the lesion length data, i.e., bacterial numbers were significantly reduced through the effects of the resistance genes at each plant age.

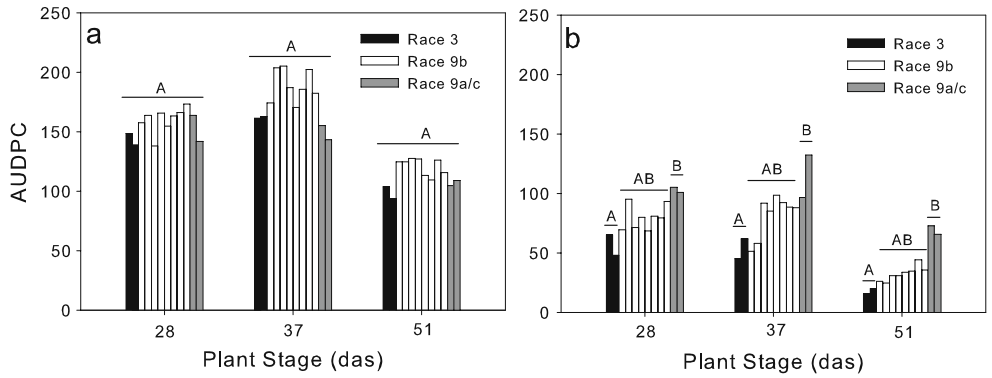

Fig. 5 **a** and **b** Influence of *avrXa7* allele on AUDPC after inoculation of rice cultivars IR24 **a** and IRBB7 **b** at different plant stages (*das*). Plants at 28 das are at the two leaf (*seedling*) stage, plants at 37 das are at the maximum tillering, and plants at 51 das are at booting stage. Black bars are race 3 strains (PXO1865, PXO368); white bars, race 9b strains (PXO433, PXO356, PXO441, PXO448, PXO357, PXO419, PXO348, PXO0314); gray bars, race 9a/c strains (PXO346, PXO2684) respectively. Capital letters indicate significant differences in lesion lengths within a plant growth stage (all p-values<0.001)

However, in the case of *Xa4*, although lesion length data indicated resistance, bacterial numbers were not significantly lower in plants at all growth stages (particularly at 41 das). The mechanisms of resistance are not known for any of the interactions investigated; however, it is possible that *Xa4* mediated resistance may limit pathogen movement down the leaf while allowing *Xoo* to still multiply in the host. Iyer-Pascuzzi et al. (2008) previously noted that in *xa5* interactions, bacterial numbers will multiply to high levels in resistant lines (with *xa5*) but that bacterial movement down the leaf is restricted. Earlier reports also indicate that plants with *Xa4* support high numbers of bacteria in incompatible interactions, even approaching levels in susceptible interactions, but show shorter lesions than susceptible interactions (Leach et al. 2001). Furthermore, incompatible interactions with the *Xa4* gene do not exhibit a classic hypersensitive response (Guo et al. 1993). *Xa4* has been suggested to confer partial resistance (Vera Cruz and Mew, pers. commun.), and has even been suggested to exhibit quantitative rather than qualitative action (Li et al. 2001). Understanding how *Xa4* and other *R* genes impact *Xoo* multiplication and spread in rice leaves are interesting questions needing resolution for efficient and effective *R* gene deployment.

In addition to a specific growth age (i.e. days after planting), the particular leaf position on the rice plant, which is a reflection of specific plant development, may impact *R* gene expression. For example, Bai (1990) determined that while susceptible lesion types did not vary between different leaf positions, lesion types during resistant interactions did vary with leaf age. In his study, interactions involving *Xa10* showed no difference in lesion types at different leaf positions, but the interactions involving *Xa7* exhibited a more restricted lesion in the older leaf positions (Bai 1990). Others have also demonstrated that resistance is not only dependent on the age of the plant but also on the age or position of individual leaves (Noda and Ohuchi 1989). We did not assess influence of leaf position in this study.

Resistance conferred by *xa5* has been anecdotally reported to be highly variable in immature plants, however we did not find any developmental regulation of function. Among the BB *R* genes, *xa5* is unique in that it is inherited recessively and encodes a eukaryotic transcription factor (Iyer-Pascuzzi and McCouch 2004; Iyer-Pascuzzi et al. 2008). How the *xa5* protein interacts in different genetic backgrounds to result in resistance may be distinct from other *R* genes, and this could be associated with the high variability observed in other studies. However, to critically evaluate this, more quantitative assays run under different environmental conditions and genetic backgrounds should be performed.

There may be other factors in addition to plant age, which could influence the effectiveness of particular plant *R* genes including temperature, rainfall or relative humidity, and agronomic conditions. Webb et al. (2010) found that *Xa7* is more effective at high temperatures, whereas other *Xa* genes are less effective at high temperatures (i.e. *Xa4, xa5,* etc). Agronomic practices, particularly crop fertilization

levels may influence *R* gene function. Bai (1990) demonstrated that high nitrogen levels made rice plants with *Xa4* and *Xa7* more susceptible to bacterial blight (even in incompatible interactions), but saw no change in *xa5* resistance. He predicted that the enhanced susceptibility was in part related to increased plant biomass.

Based on the predicted durability of these particular *R*-genes (Bai et al. 2000; Vera Cruz et al. 2000) and their effectiveness at all stages of growth, these would be good candidate genes for incorporation into commercial cultivars. *Xa4* has already been deployed in commercial cultivars, and has proven to be durable, likely in part due to its effectiveness at all developmental growth stages as we have shown here. While seedling resistance is important for deployment, screening for resistance should be performed at older growth stages (after at least 21 das) to determine the most practical results.

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