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SHORT COMMUNICATION

## Developmental control of *Xa21*-mediated disease resistance in rice

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### Summary

The rice resistance gene *Xa21* confers resistance against the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (Xoo). The molecular genetic mechanism controlling the integration of the *Xa21*-mediated disease resistance response with the developmental program in rice is under study in this model system. Reproducible means of infecting plants at certain developmental stages were designed based on the timing of full expansion of the leaf. *Xa21*-resistance progressively increases from the susceptible juvenile leaf 2 stage through later stages, with 100% resistance at the adult leaf 9/10 stage. We found that *Xa21* expression is independent of plant developmental stage, infection with Xoo, or wounding. Expression of the *Xa21* gene transcript is not correlated with expression of *Xa21* disease resistance indicating that the developmental regulation of *Xa21*-resistance is either controlled post-transcriptionally or by other factors.

### Introduction

In many plant–pathogen interactions involving both monocots and dicots, as well as various bacterial, fungal and viral pathogens, resistance to disease is developmentally controlled. Because of the developmentally limited protection afforded to the host plant, resistance expressed at one particular stage is less desirable agronomically than that expressed at all stages in growth. Other than descriptions of the phenomenon, only a few studies have

addressed the mechanistic basis of the developmental control of disease resistance in plants. One particularly illustrative study examined the maize mutation called corngrass (Cg) in which expression of adult stage characteristics is developmentally delayed (Poethig, 1988). Resistance to common rust in maize is developmentally controlled and normally expressed only at adult stages. In Cg mutant lines, resistance is delayed co-ordinately with the delay of adult leaf characteristics (Abedon and Tracy, 1996). In this case, disease resistance was controlled by genes that control the transition from juvenile to adult developmental phases. This is the only known study that clearly addresses the interaction of developmental change and disease resistance at the genetic level.

In various rice lines, resistance to both *Magnaporthe grisea*, the causal agent of rice blast disease, and *Xanthomonas oryzae* pv. *oryzae* (Xoo), the causal agent of bacterial leaf blight, is developmentally controlled (Goel and Gupta, 1990; Kim *et al.*, 1987; Mew *et al.*, 1981; Mew, 1987; Ogawa, 1993; Yeh *et al.*, 1989; Zhang and Mew, 1985). In general, in rice adult plants, resistance exhibits race- and cultivar-specificity and is affected by environmental conditions. At later growth stages, resistance against all isolates usually increases in a race non-specific manner (Mew, 1987). Studies of the bacterial blight resistance response conditioned by the resistance gene *Xa21* indicate that *Xa21*-mediated resistance to Xoo is not expressed in the early stages of development (Mazzola *et al.*, 1994). However, after 21 days post-germination, the *Xa21* line exhibits resistance, and as the plant matures further, resistance continues to increase.

The *Xa21* gene encodes a receptor-like protein kinase suggestive of a role in cell surface recognition of a pathogen ligand and subsequent activation of an intracellular kinase leading to a defence response (Song *et al.*, 1995). *Xa21* is the only known resistance gene that encodes three structural features found in various combinations in other resistance gene products: an extracellular domain with leucine rich repeats (LRR), a transmembrane domain, and a cytoplasmic serine/threonine kinase domain. As such, *Xa21* is unique among resistance genes of plants. While this combination of structural features is lacking in other cloned resistance genes, three developmental genes of *Arabidopsis* are structurally similar to *Xa21*: *CLV1*, *ERECTA1* and *BRI1* involved in shoot apical meristem development, organ shape and growth regulator

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perception, respectively (Clark *et al.*, 1997; Li and Chory, 1997; Torii *et al.*, 1996).

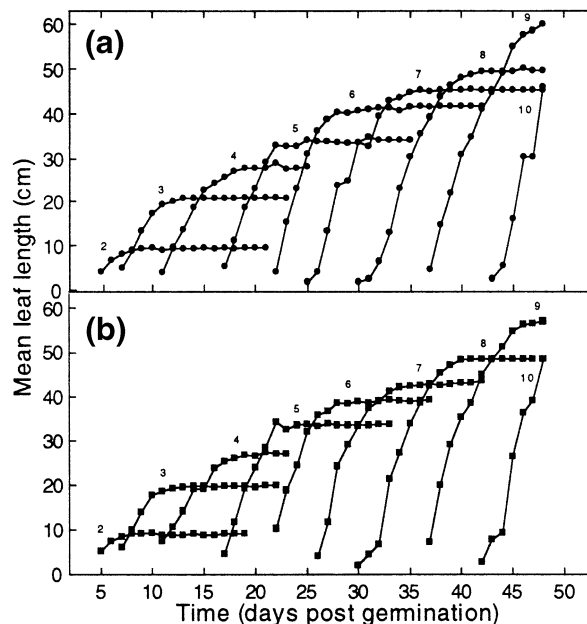
The *Drosophila* LRR receptor Toll provides a model for examining the molecular genetic linkage between development and disease resistance (Lemaitre *et al.*, 1996; reviewed in Wilson *et al.*, 1997). Several components of the Toll signaling pathway are involved in both development and pathogen defence. Interestingly, Toll signaling pathway homologues have been found to play defence-related roles in plants (Baker *et al.*, 1997). More information is required to understand the connection of disease resistance and development in all of these systems.

The Xoo–*Xa21* interaction provides a model system in which to study the molecular basis of developmental control of disease resistance because the *Xa21* gene has been cloned (Song *et al.*, 1995). Understanding the molecular genetic basis of the developmental regulation of disease resistance will allow for the design of strategies which increase the usefulness of stable resistances that are only expressed later in development. In the studies described here, we address the mechanistic basis of developmentally controlled disease resistance in rice. We describe a reproducible means of assessing the physiological age of rice plants. We assessed lesion length in just fully expanded leaves and found that *Xa21*-resistance increases over time from juvenile leaf 2 stage through later stages. To test the hypothesis that this developmental expression of disease resistance results from developmental expression of the *Xa21* gene, we analyzed levels of the *Xa21* transcript. We found that *Xa21* expression is independent of plant developmental stage, infection with Xoo or wounding. Expression of the *Xa21* gene in the leaf is not correlated with expression of *Xa21*-mediated disease resistance, indicating that resistance is either regulated at *Xa21* post-transcriptionally or that other factors are involved.

## Results and Discussion

### Leaf development in rice

Studies of developmental expression of *Xa21* resistance depend on the unambiguous determination of the physiological stage of leaves. At the time of full expansion, the base of the leaf blade just above the leaf–sheath boundary unrolls and becomes flattened. Leaf length measurements substantiated this morphological observation (Figure 1). The pattern of leaf initiation and development on the shoot is sequential in rice (Figure 1; Nemoto *et al.*, 1995), thus by using the same numbered leaf at the time just after full expansion we are able to optimize reproducibility of results from analysis of lesion length and gene expression. We conclude that the leaf number and not days post-germination is the most robust indicator of developmental



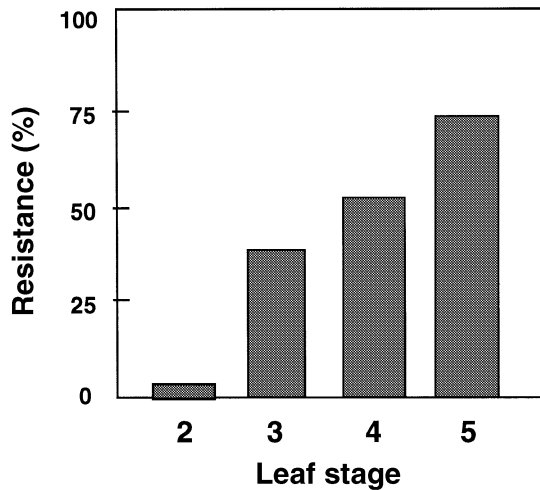
**Figure 1.** Developmental time course of the leaf length of rice lines. The length of leaves of two near-isogenic rice lines during the early developmental period was measured each day during the first 50 days post-germination and expressed as the mean. The sample size was 10 plants for each line. (a) Resistant *Xa21*-line; (b) susceptible line IR24. The numbers near each curve represent leaf number.

physiology. Morphological observations indicate that axillary shoots (tillers) emerge around the time that seedlings begin to acquire full resistance. Because of the co-ordinate timing, it is possible that further study will reveal a mechanistic relationship between resistance and tiller emergence (e.g. release of apical dominance).

### *Xa21*-resistance at different developmental stages in leaves

To learn about the response of leaves in the resistant and susceptible lines, leaves just fully expanded were infected with Xoo and subsequent lesion lengths were measured. A time course comparing lesion length in the susceptible line with that in the resistant line was performed (Figure 2). Each sequential leaf just at full expansion is more resistant than the preceding leaf, which means that acquiring the resistance phenotype is a gradual process from fully susceptible leaf 2 to leaf 5 with 75% resistance. Leaves 9 and 10 are fully resistant (Song *et al.*, 1995). Acquisition of the resistance phenotype appears to follow a spatial or temporal gradient and models of developmental control mechanisms must take into account this leaf-specific process.

To begin to understand the interaction of Xoo with host cells as disease lesions progress through leaf tissue and to develop protocols for tissue harvest for RNA isolations,



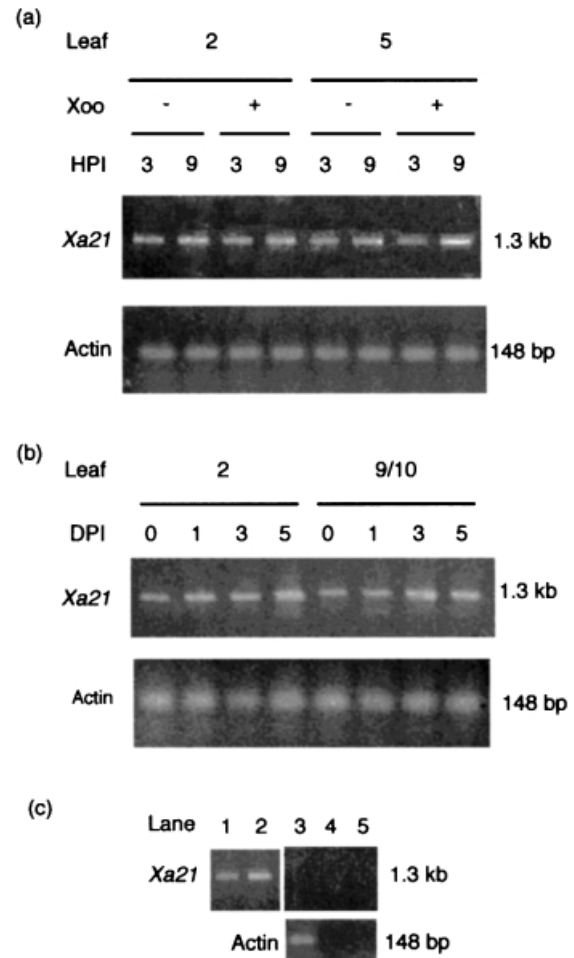
**Figure 2.** Developmental control of the *Xa21* resistance phenotype. Leaves 2, 3, 4 and 5 at just full expansion of *Xa21* line and the susceptible line IR24 were inoculated with Xoo race 6 and the resulting lesions were measured 12 days post-inoculation. The percentage resistance was calculated as 1 minus the ratio of the mean *Xa21*-line lesion length to mean susceptible line lesion length  $\times 100$ . The sample size was 12 plants per leaf stage per line.

experiments were conducted to evaluate the temporal and spatial distribution of Xoo populations. Using our routine inoculation technique and density of Xoo ( $5 \times 10^8$  colony forming units (cfu)/ml), we found that  $3 \times 10^3$  cfu are delivered to the cut surface at the tip of the leaf. In the early stages of infection (<3 h), bacteria multiply and move about 1.0 cm distal to the inoculation site. This information was used to optimize the design of the gene expression studies that follow.

#### Analysis of *Xa21* gene expression

Beginning with the simplest hypothesis that the developmental pattern of *Xa21* resistance phenotype is directly correlated with the pattern of *Xa21* gene expression, the first step was to determine if *Xa21* is expressed in both resistant and susceptible tissues. This task was complicated by the fact that *Xa21* is part of a multi-gene family with at least eight members with a high degree of identity at the DNA level family (Song *et al.*, 1997). Analysis of transgenic plants has shown that, of the seven family members tested, only *Xa21* confers strong resistance to Xoo (Wang *et al.*, 1998). Northern blots do not provide sufficient specificity to distinguish *Xa21* from other members of the closely related gene family (M. Yoshikawa and P.C. Ronald, unpublished results). Therefore, our approach was to use allele-specific reverse transcriptase-PCR (RT-PCR) and ribonuclease protection assay (RPA).

An RT-PCR strategy was developed to detect the expression of *Xa21* over a developmental time course



**Figure 3.** The analysis of *Xa21* expression by reverse transcriptase-PCR (RT-PCR) at susceptible and resistant stages.

(a) *Xa21*- or actin-specific RT-PCR was performed on RNA from leaf 2 (2% resistant) and leaf 5 (75% resistant) of the *Xa21* line. Leaves were inoculated with Xoo race 6 (+) or mock-inoculated with water (-) and RNA was isolated at 3 and 9 h post-inoculation (HPI).

(b) *Xa21*- or actin-specific RT-PCR was performed on RNA from susceptible leaf 2 and fully resistant leaves 9 and 10 of *Xa21* line. Leaves were inoculated with Xoo race 6 and RNA was isolated immediately following inoculation (0) and 1, 3 or 5 days later (DPI).

(c) The analysis of the *Xa21* expression in *Xa21* resistant line and susceptible IR24 line. Leaves were not inoculated. Lanes: (1) *Xa21*-line, leaf 2; (2) *Xa21*-line, leaf 5; (3) IR24 line, leaf 5; (4) *Xa21*-line, leaf 5 reaction with no reverse transcriptase; (5) RT reaction with no RNA.

with primers that were designed to maximize differentiation of the target *Xa21* transcript from the non-target *Xa21* family members' transcripts. Primers flanked the intron in *Xa21* to distinguish cDNA from contaminating genomic DNA. Gene expression of actin was used as a normalization control. To examine *Xa21* expression shortly after infection, RNA was isolated from 1 cm tips of leaf blades of susceptible leaf 2 and resistant leaf 5 stage plants at 3 and 9 h post-inoculation with either Xoo race 6 or water (mock inoculation) and RT-PCR was performed. A PCR product corresponding to the expected size of the amplified *Xa21*

transcript was present in all samples (Figure 3a). Sequencing of cloned RT-PCR products verified that the amplification product was *Xa21* and not other members of the *Xa21* family. This evidence suggests that at short times post-infection and post-inoculation with water, *Xa21* is expressed.

The *Xa1* resistance gene was recently cloned and shown to be induced by Xoo infection after 3 days (Yoshimura *et al.*, 1998). To assess expression of *Xa21* at longer times post-infection, RT-PCR was used to assay *Xa21* transcript in susceptible leaf 2 and fully resistant leaf 9/10 stages during a time course up to 5 days post-infection. We found an RT-PCR product corresponding to the expected size and sequence of the *Xa21* transcript at 1, 3 and 5 days post-infection (Figure 3b). In addition, the *Xa21* transcript was found when tissue was harvested immediately following infection (0 DPI, Figure 3b). In contrast to bacterial blight resistance gene *Xa1*, *Xa21* was expressed at all times in leaves. Since the *Xa1* gene appears to be induced in response to wounding (Yoshimura *et al.*, 1998), we investigated the response of *Xa21* to wounding by examining *Xa21* expression in leaves 2 and 5 that had not been treated. *Xa21* transcript was present in the uninoculated leaves, indicating that, unlike *Xa1*, *Xa21* is expressed constitutively in leaf tissue (Figure 3c). *Xa21* gene expression was not observed in the susceptible line IR24 (Figure 3c). Our results are supported by RPA results (data not shown). In summary, we found that *Xa21* is expressed in the leaf at both susceptible and resistant stages, and that expression is not dependent on infection with Xoo or wounding.

Since expression of the *Xa21* gene transcript is not correlated with phenotypic expression of *Xa21* disease resistance, developmental regulation of *Xa21* resistance is either controlled post-transcriptionally at *Xa21* or by other molecular genetic mechanisms. Post-transcriptional regulation could potentially involve mRNA stability, translational control, post-translational modification or protein turnover rate. Once XA21-specific antisera are developed, we will be able to assess XA21 protein levels in the various developmental stages. Considering that XA21 has an intracellular serine-threonine kinase domain, it is a likely possibility that XA21 activity is controlled by phosphorylation status. At the juvenile stage of development, the phosphorylation state may signal inactivity to downstream mediators of the resistance pathway, whereas at the adult stage of development it may transmit a signal that activates the resistance pathway. With XA21-specific antisera, we will be able to test this hypothesis by analyzing the phosphorylation status of XA21 in juvenile and adult stages. If developmental control of *Xa21* resistance appears not to be post-transcriptional, then it is possible that another factor confers developmental competence for the pathogen response. A screen for

precocious resistant mutants is underway to allow us to dissect pathways controlling such functions.

The fact that domains in XA21 are similar to those in the receptor kinases that control various developmental processes is intriguing and merits further study. It is interesting that the expression of the developmental gene *Clv1* does correlate with its role in development (Clark *et al.*, 1997). Moreover, it appears that the function of CLV1 and several other receptor serine-threonine kinases is controlled by a downstream phosphatase called KAPP (kinase associated protein phosphatase) (Stone *et al.*, 1998; Williams *et al.*, 1997). The binding of KAPP to CLV1 is dependent on the phosphorylation state. In contrast, a rice KAPP homologue does not interact with XA21 (van der Knaap *et al.*, 1999), so the extent of the similarity of the two systems is uncertain. The study of developmental control of the *Xa21*-mediated resistance phenotype will contribute to our understanding of the intersection of disease resistance and development.

## Experimental procedures

### Bacterial maintenance

*Escherichia coli* DH5 $\alpha$  was subcultured at 37°C on Luria medium (Miller, 1972). *Xanthomonas oryzae* pv. *oryzae* race 6 PX099A (Xoo) (Hopkins *et al.*, 1992) was used for pathogen infections. Xoo was subcultured at 30°C on PSA (1% peptone, 1% sucrose, 0.1% glutamate; Tsuchiya *et al.*, 1982). Bacto agar at 1.5% (wt/vol) was added to media for plate cultures. Antibiotics (Sigma, St. Louis, MO, USA) were used for selection at the following concentrations in  $\mu\text{g ml}^{-1}$ : ampicillin (Ap), 50; kanamycin (Km), 25.

### Plant maintenance and pathogen infection

Rice seeds were imbibed overnight at 30°C and placed on moist filter paper overnight at 30°C. Germinated seeds were then sown in UC potting mix. The chronological age of the plant is based on the number of days post-germination, with germination referring to the day the germinated seeds were planted. Pots with plants were kept in standing nutrient solution (0.25 $\times$  Hoagland's solution) in growth rooms within a greenhouse with high intensity plant growth lights providing a supplemental 12 h photoperiod.

Leaves were infected with Xoo at an OD600 of 0.5 (approximately  $5 \times 10^8$  cfu ml $^{-1}$ ) by using scissors dipped in bacterial suspensions to clip leaves 1–2 cm down from the tip of the leaf blade (Kauffman *et al.*, 1973). Mock-infected plants were treated in a similar fashion except that water was used. Leaves were inoculated within 1 day of reaching full expansion, defined as the stage at which the next leaf appears and the distal portion of the leaf blade flattens out from a rolled configuration. After infection, plants were placed in a growth chamber (32°C day, 22°C nights, 12 h photoperiod). Lesion length was measured from the cut surface at the tip to the distal-most position on the leaf that exhibits a grey, chlorotic or water-soaked lesion. We adhere to regulations on the use of Xoo based on the APHIS pathogen permit from the California State Department of Agriculture.

### Nucleic acid manipulation

Standard molecular biology techniques were used (Ausubel *et al.*, 1997; Kreig, 1996; Sambrook *et al.*, 1989). For mini-preparations of RNA, RNeasy (Qiagen, Valencia, CA, USA) spin columns with added DNase treatment step according to the manufacturer's instructions were used. For large preparations of RNA, a standard LiCl isolation protocol was used (Kreig, 1996). The purity, yield and intactness of the RNA were assessed by spectrophotometry and formaldehyde gel electrophoresis. Ribonuclease protection assay (RPA) was performed using RPAII kit from Ambion (Austin, TX, USA).

### Reverse-transcriptase PCR

For RT-PCR, RNA was isolated from 1 cm tips of inoculated leaf blades at specific time points post-inoculation. For the analysis of *Xa21* and actin transcripts, total RNA (3 µg) was heat denatured and added to a reverse transcriptase (RT) reaction containing 1× RT buffer, 10 mM DTT, 0.5 mM each dNTP, first-strand primer (1 µM each dT<sub>12</sub>VG, dT<sub>12</sub>VT, dT<sub>12</sub>VC, and dT<sub>12</sub>VA), and 200 units Superscript II (Gibco BRL, Gaithersburg, MD, USA). The resulting cDNA was then treated with 2 units RNase H (Gibco BRL) at 37°C for 20 min. PCR was carried out using standard conditions (1 unit Taq polymerase, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.1 mM dNTPs, 0.25 µM forward primer, 0.25 µM reverse primer). Two µl (10%) of each reverse transcription reaction were used per PCR sample. For amplification controls, *Xa21* genomic DNA, *Xa21* cDNA clone pCR504 (M. Chern and P.C. Ronald, unpublished results) and rice actin cDNA clone pAc1 (McElroy *et al.*, 1990) were used as templates. *Xa21*-specific primers were 5'-ATTGCCAGCTTACGGT-3' and 5'-CAAGTCTAAGCAGCC-AAACA-3', and primers for actin were derived from Yoshimura *et al.* (1998). The PCR program consisted of an initial denaturation for 5 min at 95°C, 30–35 cycles of 45 sec at 95°C, 45 sec at 58°C, 2 min at 72°C, then a final extension step of 5 min at 72°C. It was not possible to amplify *Xa21* and actin in the same reaction. Cycling conditions were optimized to allow for the detection of a range of concentrations of *Xa21* cDNA clone pCR504, and the *Xa21* RT-PCR product was within that range. An equal volume of each PCR sample was fractionated on 1.5% agarose gels.

To verify specific amplification of *Xa21*, PCR products from seven reactions were cloned into vector pPCRII using Invitrogen TA cloning kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Two µg of three individual clones from each sample were purified using the Qiaprep miniprep system (Qiagen) and sequenced by the dideoxy chain-termination method using M13 forward and M13 reverse primers (ABI Prism Dye Primer Cycle Sequencing Kit and ABI Prism Model 377 Sequencer; Perkin Elmer, Norwalk, CT, USA). Resulting sequences were analyzed by the Sequencher (Gene Codes) software.

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