

Rice *xa13* Recessive Resistance to Bacterial Blight Is Defeated by Induction of the Disease Susceptibility Gene *Os-11N3* ^{WJ}^{OA}

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The rice (*Oryza sativa*) gene *xa13* is a recessive resistance allele of *Os-8N3*, a member of the *NODULIN3* (*N3*) gene family, located on rice chromosome 8. *Os-8N3* is a susceptibility (*S*) gene for *Xanthomonas oryzae* pv *oryzae*, the causal agent of bacterial blight, and the recessive allele is defeated by strains of the pathogen producing any one of the type III effectors *AvrXa7*, *PthXo2*, or *PthXo3*, which are all members of the transcription activator-like (TAL) effector family. Both *AvrXa7* and *PthXo3* induce the expression of a second member of the *N3* gene family, here named *Os-11N3*. Insertional mutagenesis or RNA-mediated silencing of *Os-11N3* resulted in plants with loss of susceptibility specifically to strains of *X. oryzae* pv *oryzae* dependent on *AvrXa7* or *PthXo3* for virulence. We further show that *AvrXa7* drives expression of *Os-11N3* and that *AvrXa7* interacts and binds specifically to an effector binding element within the *Os-11N3* promoter, lending support to the predictive models for TAL effector binding specificity. The result indicates that variations in the TAL effector repetitive domains are driven by selection to overcome both dominant and recessive forms of resistance to bacterial blight in rice. The finding that *Os-8N3* and *Os-11N3* encode closely related proteins also provides evidence that *N3* proteins have a specific function in facilitating bacterial blight disease.

INTRODUCTION

Plants have evolved mechanisms that protect against pathogen effector-mediated susceptibility of which the resistance (*R*) genes are an important component (Chisholm et al., 2006; Ellis et al., 2009). *R* gene products have been proposed to guard important defense signaling complexes that are targeted by virulence effectors by sensing perturbations upon the interaction of the complex with a pathogen virulence effector or, alternatively, by acting as target decoys, intercepting effectors upon their entry into the host (Hogenhout et al., 2009). In either event, perception triggers rapid defense responses that are typically associated with localized cell death, commonly known as a hypersensitive reaction. Bacterial pathogens can evade or defeat effector-triggered resistance by a variety of genetic changes, which include alterations in effector structure, resulting in loss of *R* gene-mediated resistance; outright loss or inactivation of cognate effector genes and loss of effector recognition; and acquisition of new effector genes that mediate suppression of *R* gene-mediated resistance. Recent evidence in rice (*Oryza sativa*) and wheat (*Triticum aestivum*) indicate that host resistance to disease also involves genetic variability in dominant traits that are targeted by virulence effectors, which we refer to here as susceptibility (*S*) genes and are commonly revealed as recessive resistance genes (Liu et al., 2009; White and Yang, 2009). In

contrast with the numerous examples of dominant *R* gene-mediated resistance, few genetic variations in effector-triggered susceptibility have been characterized (Deslandes et al., 2002; Piffanelli et al., 2004; Iyer-Pascuzzi and McCouch, 2007; White and Yang, 2009).

The recessive *R* gene *xa13* occurs as a series of natural alleles of the *S* gene *Os-8N3*, whose expression is induced by strains of *Xanthomonas oryzae* pv *oryzae* carrying the gene *pthXo1*, which encodes the transcription activator-like (TAL) effector *PthXo1* (Chu et al., 2006; Yang et al., 2006; Yuan et al., 2009). The *xa13* alleles are unresponsive to *PthXo1*, and plants with *xa13* are resistant to strains of the pathogen that rely solely on *PthXo1* as the essential effector for virulence (Yang et al., 2006). *PthXo1* is secreted via the bacterial type III secretion system and is a member of the TAL effector family, which consists of a large number of closely related nuclear-localized DNA binding proteins (White et al., 2009). TAL effectors mediate host gene expression and function as transcription factors within the host cells (Kay et al., 2007). Individual TAL effectors induced expression of specific host genes, and differences in host gene specificity are determined by the repetitive central region of each effector, which consists of direct repeats of 34- to 35-amino acid residues. The repetitive regions have been proposed to determine the sequence specificity within the promoters of the affected genes (Boch et al., 2009). *PthXo1* has 23.5 repeats and is encoded by one of 19 TAL effector genes in the genome of *X. oryzae* pv *oryzae* strain PXO99^A (Yang and White, 2004; Salzberg et al., 2008). *PthXo1* is the only effector of PXO99^A that is capable of *Os-8N3* induction, and mutants of *pthXo1* in PXO99^A are severely reduced in virulence on all otherwise susceptible rice cultivars (Yang and White, 2004).

xa13-mediated resistance is race-specific resistance, meaning that *xa13*-mediated resistance has been defeated by some

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strains of *X. oryzae* pv *oryzae* (Lee et al., 2003; Chu et al., 2006). How *xa13* is defeated is unknown. In this regard, PthXo1 is one of four known TAL effectors from different strains of *X. oryzae* pv *oryzae* that have major contributions to virulence, which we refer to as major TAL effectors (Yang and White, 2004). The major TAL effectors also include AvrXa7, PthXo2, and PthXo3, and each contain unique repetitive regions. AvrXa7 further differs among the four as the cognate effector for the dominant *R* gene *Xa7*. Furthermore, the three alternate major TAL effectors were identified in races of the pathogen that are compatible on rice lines containing *xa13*, and we previously demonstrated that introduction of the gene *avrXa7* into PXO99^A was sufficient to overcome *xa13*-mediated resistance (Yang et al., 2006). Here, we analyzed the ability of additional major TAL effectors to circumvent *xa13*-mediated resistance and attempted to identify induced host genes that circumvent the need for *Os-8N3* function in susceptibility to bacterial blight disease of rice.

RESULTS

Alternate Major TAL Effectors AvrXa7, PthXo2, and PthXo3 Do Not Induce Os-8N3

To test whether individual major TAL genes other than *pthXo1* determined compatibility of *X. oryzae* pv *oryzae* in plants with *xa13*, derivatives of PXO99^AME2, a *pthXo1* mutant derivative of PXO99^A (hereafter, ME2), containing the vector pHM1 alone or the vector with one of the major TAL effector genes *avrXa7*, *pthXo2*, or *pthXo3* were tested for virulence on IRBB13, a rice line that is derived from the recurrent susceptible parental line IR24 and homozygous for *xa13*. The allele of *xa13* in rice line IRBB13 has a 253-bp insertion/38-bp deletion within the promoter region of *Os-8N3* in comparison to IR24 (Chu et al., 2006; Yang et al., 2006). (Strains and plasmids are provided in Supplemental Table 1 online.) ME2 itself fails to form lesions on either IRBB13 or IR24 due to the lack of at least one major TAL effector gene for virulence (Figure 1A, treatment 1). Reintroduction of *pthXo1* to ME2 restored virulence on IR24 (Figure 1A, treatment 2, white column) but not on IRBB13 due to the inability of PthXo1 to induce *Os-8N3* in this line (Figure 1A, treatment 2, black column; Yang et al., 2006). Addition of *avrXa7*, *pthXo2*, or *pthXo3* to ME2 restored virulence on both IR24 and IRBB13 (Figure 1A, treatments 3 to 5, respectively). The strains were then tested for the ability to induce *Os-8N3* in either IRBB13 or IR24 as measured by quantitative RT-PCR (qRT-PCR) and RNA gel blot hybridization (Figure 2B). *Os-8N3* expression was 168-fold greater in IR24 after inoculation with ME2(*pthXo1*) compared with ME2 (Figure 1B, treatment 2, white column), and no increase in *Os-8N3* expression was detected in IRBB13 with ME2(*pthXo1*) (Figure 1B, treatment 2, black column) or any combination of rice lines with strains with the alternate TAL effectors (Figure 1B, treatments 3 to 5).

AvrXa7 and PthXo3 Induce Os-11N3, Another Member of the N3 Gene Family

Os-8N3 is one of 17 *N3* genes in rice (Yang et al., 2006), and the ability of the alternate TAL effectors to promote the expression of

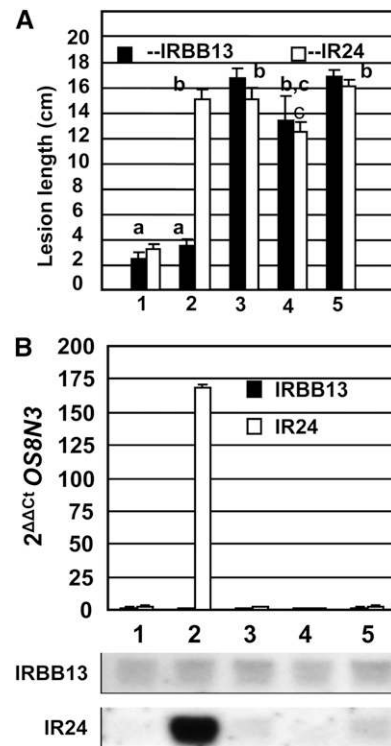


Figure 1. Major TAL Effector Genes Defeat *xa13* in Rice without Induction of *Os-8N3*.

(A) Disease leaf lesions on 10 28-d-old plants of cultivars IRBB13 (black bars) and IR24 (white bars) were measured 14 d after inoculation at the leaf tips with bacterial suspensions (0.5×10^8 colony-forming units/mL) of the following strain treatments: 1, ME2 (pHM1); 2, ME2 (*pthXo1*); 3, ME2 (*pthXo2*); 4, ME2 (*pthXo3*); 5, ME2 (*avrXa7*). Values with the same lowercase letters above columns do not differ significantly at the <0.5 level using Tukey statistic and ANOVA analysis. Bars indicate 1 SD.

(B) qRT-PCR measurements of *Os-8N3* expression following strain treatments as for **(A)**. RNA was prepared 24 h after inoculation of leaves, and qRT-PCR was performed using gene-specific primers for rice locus *Os-8N3*. Values are normalized against the expression of the rice gene *TFIIA γ 5* (NM 001060961). RNA gel blot hybridization analysis of *Os-8N3* following bacterial inoculation is shown below the qRT-PCR data for each treatment. Total RNA was prepared from 10 leaves of rice cultivars IRBB13 and IR24 and measured spectrophotometrically. Equal amounts from each treatment were loaded in an agarose gel, fractionated, and subjected to hybridization analysis using ³²P-labeled fragment of *Os-8N3*.

other members of the *N3* gene family in infected rice leaves was examined. cDNA was prepared from leaf mRNA after individual inoculations of cultivar Nipponbare with strains ME2, ME2(*avrXa7*), ME2(*pthXo2*), and ME2(*pthXo3*) and subjected to qRT-PCR using gene-specific primers derived from the 3'-untranslated region (UTR) sequences of *N3* gene family members, starting with the members most similar to *Os-8N3* (Yang et al., 2006). The gene *Os11g31190* (hereafter, *Os-11N3*) was induced both in an AvrXa7- and PthXo3-dependent manner (Figure 2A, treatments 6 and 7). Control cDNA samples were also prepared from uninoculated leaves, mock-inoculated (water) leaves, and leaves inoculated with bacteria deficient in type III secretion (ME7), ME2

(*pthXo1*), and ME2(*pthXo2*). All failed to induce *Os-11N3* (Figure 2A, treatments 1 to 5). ME2(*pthXo3*) induced *Os-11N3* an average of 71-fold over uninoculated plants (Figure 2A, treatment 6), while ME2(*avrXa7*) induced *Os-11N3* 52-fold over ME2 (Figure 2A, treatment 7).

Os-11N3 is represented in databases by a 1494-base full-length cDNA (National Center for Biotechnology Information accession number AK101913), has four introns, and a predicted coding frame of 909 bp (Figure 2B). BLAST analysis was performed with the predicted protein product of *Os11N3* (Os-11N3), and 18 of the most similar proteins from monocotyledonous species and three sequences of the most similar proteins from representative dicotyledonous species were subjected to phylogenetic analysis (Figure 3). While closely related to Os-8N3, Os-11N3 is a member of a distinct clade of N3 proteins (clade II) that are separated from the Os-8N3 clade (clade I) prior to the divergence of dicots and monocots as some members from *Arabidopsis thaliana* (At5g23660), pepper (*Capsicum annuum*; CaUPA16), and soybean (*Glycine max*; GmABT17358) are more similar to Os-11N3 (Figure 3). Os-11N3 is more closely related to another clade represented by the rice *N3* gene *Os12g0476200*. The separation of Os-11N3 from *Os12g0476200* occurred prior to the divergence of rice, sorghum (*Sorghum bicolor*), and maize (*Zea mays*;

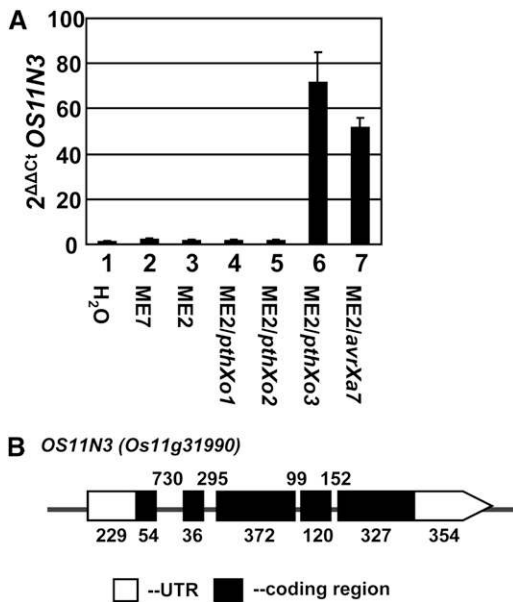


Figure 2. PthXo3 and AvrXa7 Induce *Os-11N3*.

(A) qRT-PCR analysis of *Os-11N3* expression from RNA prepared 24 h after inoculation of leaves of cultivar Nipponbare using gene-specific primers for rice locus *Os11g31990* (*Os-11N3*). Strains used in each inoculation are indicated below each lane. *TFIIA γ 5* expression was used as an internal control for the quantity and quality of RNA sample. Strains for each treatment were as follows: 1, water; 2, ME7; 3, ME2(pHM1); 4, ME2 (*pthXo1*); 5, ME2(*pthXo2*); 6, ME2(*pthXo3*); 7, ME2(*avrXa7*). RNA was extracted 24 h after inoculation. Error bars indicate 1 sd.

(B) Schematic of cDNA AK101913 corresponding to *Os-11N3* aligned with genomic sequence. Numbers indicate the bases in the indicated region.

Figure 3). The separation of Os-S11N3 from the other members within rice indicates a possible specialization of this protein in plant development or environmental responses.

Loss or Suppression of *Os-11N3* Expression Results in Loss of TAL Effector-Specific Susceptibility in Rice

The T-DNA insertion event PFG_3D-03008 was previously reported to have occurred within the first intron of *Os-11N3* in rice cultivar Hwayoung (Jeong et al., 2006). The line containing the insertion was genotyped using primers that were derived from sequence on either side of the insertion within the wild-type locus (Figure 4A, black and red arrows) and a third derived from within the T-DNA element (Figure 4A, blue arrow). One combination of primers amplified a 440-bp product from just within the right T-DNA border to the right of the insertion site (Figure 4A, PCR1), and the second set amplified a 563-bp product across the wild-type locus (Figure 4A, PCR2). A heterozygous plant was self-crossed, and the progeny were genotyped for the presence and absence of the T-DNA. All three PCR patterns, indicative of homozygous T-DNA insertion, homozygous wild type, and heterozygous, were observed (Figure 4B, examples in lanes 1, 5, and 6, respectively). All plants homozygous for the insertion, as indicated by the single PCR product specific for the T-DNA/*Os-11N3* boundary (Figure 2B, lanes 1 to 4 and 8), were resistant to ME2(*avrXa7*) and ME2(*pthXo3*) (Figure 4B, phenotype R). Heterozygous plants (Figure 4B, lanes 6, 7, and 9), homozygous wild-type plants (Figure 4B, lanes 5 and 10), and the parent preinsertion line (Figure 4B, lane 11) were susceptible to infection by ME2(*avrXa7*) (Figure 4B, phenotype S). Average lesion length measurements were obtained from six homozygous insertion plants and six heterozygous progeny with the PF_3D-03008 event after inoculation with either ME2(*pthXo1*), ME2(*avrXa7*), or ME2(*pthXo3*) (Figure 4C). Heterozygous and homozygous plants were susceptible to infection by ME2(*pthXo1*) (Figure 4C, treatment 1), while only heterozygous plants were susceptible to ME2 (*avrXa7*) and ME2(*pthXo3*) (Figure 4C, treatments 2 and 3, respectively). Homozygous plants have normal-appearing lesion phenotypes with ME2(*pthXo1*) (Figure 4D, leaf 1) and almost no lesion phenotypes with ME2(*avrXa7*) or ME2(*pthXo3*) (Figure 4D, leaves 2 and 3, respectively). Although normal in appearance, plants homozygous for T-DNA insertion are small seeded (Figure 4D, top right panel) and delayed in growth, requiring ~30 more days to reach the size of 14-d-old heterozygous (normal) plants (Figure 4D, bottom right panel). The average weight of 25 seeds from heterozygous plants was 571.48 ± 26.39 mg and that of homozygous plants 313.02 ± 11.62 mg.

The requirement for *Os-11N3* in AvrXa7- and PthXo3-mediated virulence was also assessed by RNA-mediated gene silencing (RNAi). Transgenic rice plants were generated that expressed a unique 341-base portion of the 3'-UTR of *Os-11N3* as a small double-stranded RNA to initiate silencing of the full transcript. Two plants were selected that showed high expression of the double-stranded RNA construct alone based on qRT-PCR of the 341-bp 3'-UTR fragment (Figure 5A, columns 1 and 2, black) in comparison to the plant with only vector sequences (Figure 5A, column V, black). Both plants failed to show induction of *Os-11N3* upon inoculation with ME2(*avrXa7*) based on amplification of the

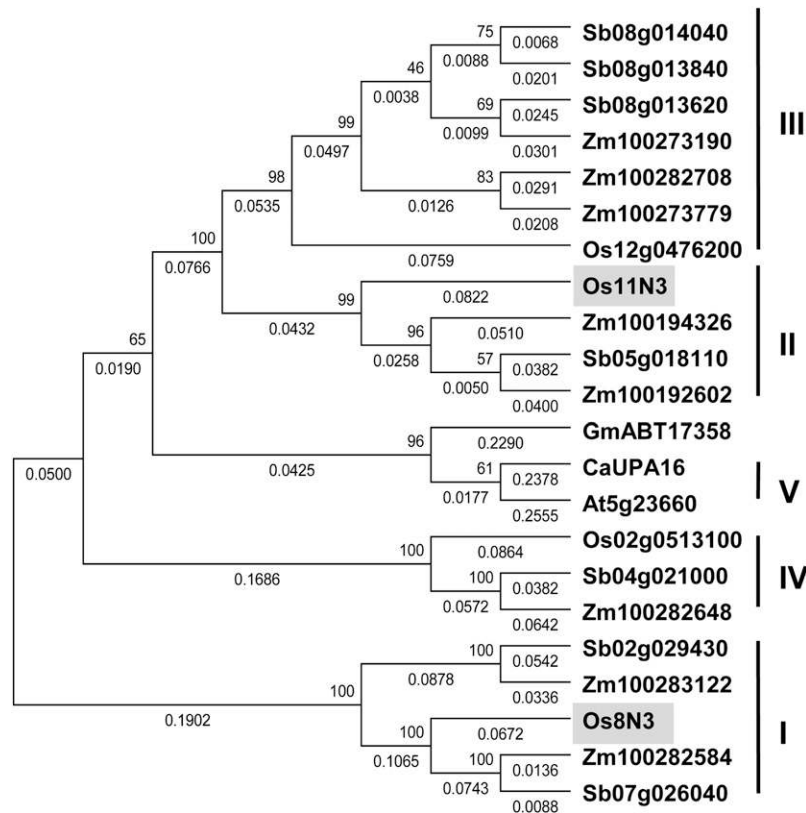


Figure 3. Rice Os-11N3 Represents a Distinct Clade of the N3 Family.

Alignment and phylogenetic analyses were conducted using ClustalW (Thompson et al., 1994) and the Minimal Evolution program in MEGA version 4 for unrooted phylogeny tree construction (Tamura et al., 2007). Bootstrap support for 1000 reiterations is provided above each line.

5'-UTR region (Figure 5A, columns 1 and 2, white). Induced Os-11N3 expression was observed in the infected control plants (Figure 5A, column V, white). Quantitative measurements based on lesion lengths following inoculation of progeny plants indicated that the control plants were equally susceptible to ME2 (*avrXa7*) (Figure 5B, column V, black) and ME2(*pthXo1*) (Figure 5B, column V, white). The progeny from the RNAi lines 1 and 2, however, had short lesion lengths upon infection by ME2(*avrXa7*), indicative of loss of the susceptibility phenotype (Figure 5B, columns 1 and 2, black), while remaining fully susceptible to ME2(*pthXo1*) (Figure 5B, columns 1 and 2, white). RNAi plants were scored visually as resistant to infection by ME2(*avrXa7*) and ME2(*pthXo3*), while control plants were susceptible (Figure 5C, showing phenotype of line 1 only). The RNAi lines remained susceptible to ME2(*pthXo1*) (Figure 5C).

Os-11N3 Promoter Contains Candidate Effector Binding Elements for *AvrXa7* and *PthXo3*

As members of the TAL effector family, *PthXo1*, *PthXo3*, and *AvrXa7* are predicted to bind effector binding elements (EBEs) in gene promoters and drive expression of their respective S genes. The consensus EBEs for *PthXo1*, *PthXo3*, and *AvrXa7*, as previously noted, was predicted from the order of the 12th and 13th

repeat residues of the protein (Figure 6A; Boch et al., 2009). The promoter regions of Os-11N3 and Os-8N3 are distinct in comparison to each other (Figure 6B). The candidate site in the Os-8N3 promoter region lies upstream of the TATA box (Figure 6B). The element overlaps the 243-bp insertion/deletion within the Os-8N3 promoter that occurs in the IRBB13 allele of *xa13* (Figure 6B). The predicted promoter region for Os-11N3 is based on the first base of the full-length cDNA. A consensus TATA box lies 25 bases upstream of the predicted transcription start site (Figure 6C). The start sites of transcription of Os-11N3 during infection with ME2 (*avrXa7*) or ME2 without *avrXa7* were analyzed by 5' rapid amplification of cDNA ends (5'-RACE-PCR). Despite low expression of the locus in normal plant leaves, six 5'-RACE cDNAs were obtained from ME2-treated tissue, and three of six began at the A located 31 residues from the TATA box and the same base as predicted from the full-length cDNA AK101913 (Figure 6C). The remaining three cDNAs have unique start sites (see Supplemental Figure 2 online). None of 13 5'-RACE cDNAs obtained from ME2(*avrXa7*)-treated tissue started at A31. Seven of 13 cDNAs, and the largest class, started at the G located 65 residues downstream from the TATA box (Figure 6C), indicating that *AvrXa7* may alter the normal transcription start site of Os-11N3. The remaining six cDNAs fell into five different classes of start sites (see Supplemental Figure 2 online). (An alignment of all cDNAs is presented in Supplemental

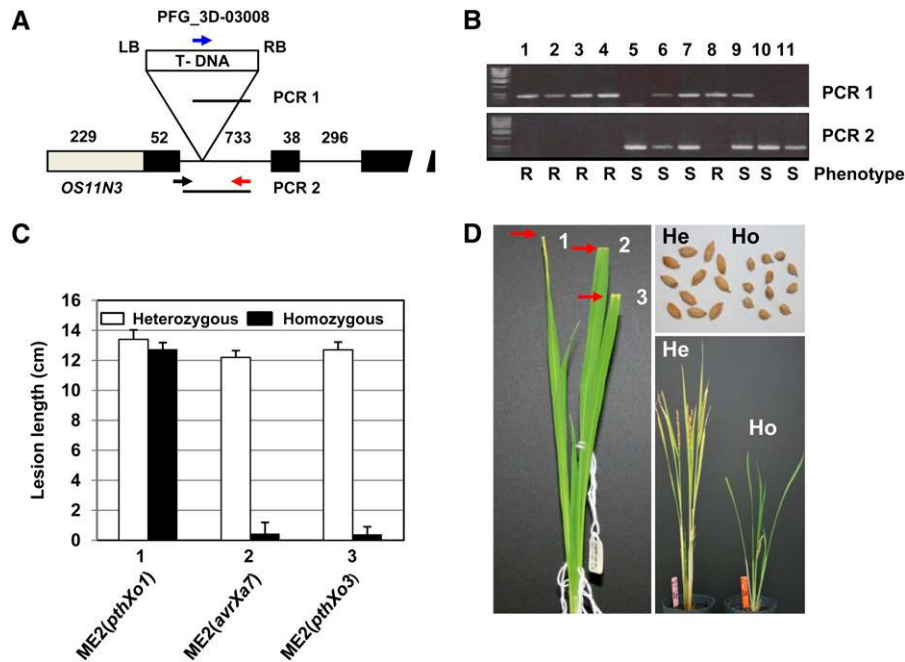


Figure 4. A T-DNA Insertion in *Os-11N3* Confers AvrXa7- and PthXo3-Specific Recessive Resistance.

(A) Position of T-DNA insertion PFG_3D-03008 within the first intron of *Os-11N3*. Schematic is not to scale. PCR product across the right border (RB) of insertion is indicated by blue and red arrows (PCR1). PCR product of the wild-type locus is indicated by the black and red arrows (PCR2). LB, left border.

(B) PCR analysis of progeny of rice cultivar Hwayoung with T-DNA insertion PFG_3D-03008. Homozygous mutant progeny are indicated by presence of fragment in the top panel (PCR1) and absence of fragment in the bottom panel (PCR2). The presence of both fragments is indicative of a heterozygous individual. The absence of PCR1 is indicative of a homozygous wild-type locus. The template for sample in lane 11 was prepared from the parent line Hwayoung. The phenotype of the line whose genotype is shown is indicated below lanes. R, resistant to infection by ME2(*avrXa7*) and ME2(*pthXo3*); S, susceptible to infection by ME2(*avrXa7*) and ME2(*pthXo3*).

(C) Average lesion length measurements of six heterozygous (white) and six homozygous plants (black) after inoculation with (1) ME2(*pthXo1*), (2) ME2(*avrXa7*), or (3) ME2(*pthXo3*). Error bars indicate 1 SD.

(D) Phenotypes of homozygous T-DNA insertion mutant inoculated with the following strains: leaf 1, ME2(*pthXo1*), leaf 2, ME2(*avrXa7*); and leaf 3, ME2(*pthXo3*). Phenotype of a homozygous T-DNA insertion mutant (plant 10-3, genotype analysis in [B], lane 8) after inoculation with the following strains: leaf 1, ME2(*pthXo1*); leaf 2, ME2(*avrXa7*); and leaf 3, ME2(*pthXo3*). Panel at left shows leaves on intact 50-d-old plant. Red arrows indicate sites of inoculation. Phenotypic differences in seed sizes are shown in the top right panel: He, heterozygous plant; Ho, homozygous mutant plant. The bottom right panel shows stature comparison of 90-d-old heterozygous and homozygous mutant individuals.

Figure 2 online.) A consensus EBE for AvrXa7 lies in the *Os-11N3* promoter within the EBE for PthXo3, starting at the second A base and ending at the second to last base of the EBE for PthXo3, and both encompass the TATA box (Figure 6C).

PthXo1 and AvrXa7 Drive Promoter-Specific Expression of *Os-8N3* and *Os-11N3*

Agrobacterium tumefaciens-mediated transient expression was used to determine if PthXo1, AvrXa7, and PthXo3 can drive *S* gene promoter-specific expression of a reporter gene in *Nicotiana benthamiana* leaves and whether the consensus EBEs discriminate between the two effectors. Promoter proximal sequences of *Os-8N3* and *Os-11N3* were fused at the start codons to the coding sequence of the *uidA* gene (β -glucuronidase [GUS]). Four promoter constructs were prepared for *Os-11N3*, including the wild type, mutant, and two hybrid constructs where the EBE for PthXo1 in the *Os-8N3* promoter was replaced with

the wild type or mutant EBE for AvrXa7 and PthXo3 (Figure 7A). Three additional *Os-8N3* promoter constructs were prepared, including the wild-type sequence, a mutant version, and the version found in the *xa13* allele in IRBB13 (Figure 7A). Expression was monitored by histological staining for GUS activity (Figure 7B), and average GUS activity was measured using the fluorescence substrate 4-methylumbelliferyl- β -D-glucuronide (MUG) from excised leaf tissue (Figure 7C).

AvrXa7 induced strong GUS activity with the wild type *Os-11N3* promoter fragment (Figure 7B, left site 1; Figure 7C, promoter 1, gray column). PthXo3 also induced activity at a lower level based on enzyme activity assays (Figure 7C, promoter 1, white column). Replacement of CCC with GGT within the overlapping EBE region for AvrXa7 and PthXo3 resulted in the loss of GUS activity for both AvrXa7 (Figure 7B, left site 2; Figure 7C, promoter 2, gray) and PthXo3 (Figure 7C, promoter 2, white). The hybrid *Os-11N3/Os-8N3* promoter fragment, containing the overlapping EBE for AvrXa7 and PthXo3 from *Os-11N3* in place of the EBE for PthXo1 (Figure

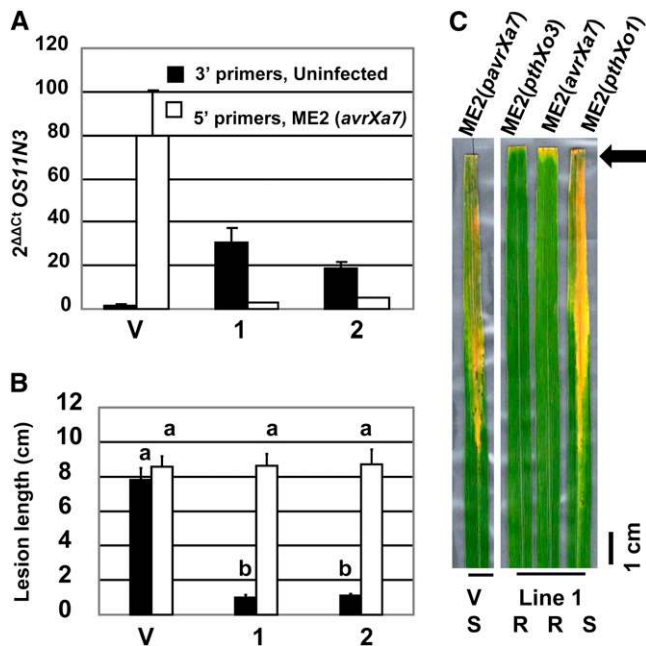


Figure 5. RNAi Knockdown of *Os-11N3* Provides *AvrXa7*- and *PthXo3*-Specific Resistance.

(A) qPCR analysis of two transgenic rice lines (1 and 2) expressing a portion of the *Os-11N3* 3'-UTR as a double-stranded RNA. A control line was also examined containing only the vector T-DNA sequences (V). RNA was prepared from plants generated without the insert (column V, vector alone) and two transgenic lines with the insert (columns 1 and 2). Black columns indicate analysis of RNA from uninfected plants, and expression of sequences from the overexpressed double-stranded *Os-11N3* 3'-UTR was amplified using 3'-specific primers. White columns indicate analysis of *Os-11N3* 5'-UTR region 24 h after inoculation of the same lines with ME2(*avrXa7*).

(B) Lesion lengths were measured 9 d after inoculation of lines V, 1, and 2 with ME2(*pthXo1*) (white) or ME2(*avrXa7*) (black). Measurements are averages of 10 plants. Values with same letter do not differ significantly at the $P < 0.5$ level using the Tukey statistic following ANOVA analysis. Error bars indicate 1 SD.

(C) Phenotypes of progeny of RNAi line 1 challenged with ME2(*pthXo1*), ME2(*pthXo3*), or ME2(*avrXa7*). Line V, containing only vector sequences, is shown after inoculation with ME2(*avrXa7*). S, susceptible; R, resistant. Arrow indicates site of inoculation. Plants were photographed 9 d after inoculation.

7A, promoter 3), resulted in *AvrXa7*- and *PthXo3*-dependent expression of GUS (Figure 7B, left site 3; Figure 7C, promoter 3, gray and white, respectively) and loss of *PthXo1*-dependent expression (Figure 7B, right site 3; Figure 7C, promoter 3, black). Inclusion of the mutant *Os-11N3* EBE in the hybrid (Figure 7A, promoter 4) resulted in the loss of both *AvrXa7*- and *PthXo3*-mediated expression of GUS activity (Figures 7B and 7C, promoter 4, black). GUS activity was observed with the wild-type (*Os-8N3pWT*) *Os-8N3* promoter when coinoculated with 35S-*pthXo1* (Figure 7B, right site 5; Figure 7C, promoter 5, black). A mutant version of the *Os-8N3* EBE (Figure 7A, promoter 6) or the promoter fragment from IRBB13 (Figure 7A, promoter 7) was unable to support GUS expression (Figure 7C, promoters 6 and 7, respectively, black).

AvrXa7 Binds to the Promoter of *Os-11N3*

Previously, *AvrBs3* was shown to preferentially bind the EBEs derived from *AvrBs3* upregulated (UPA) genes and that binding is likely to occur within the plant cell. To determine if *AvrXa7* preferentially binds the consensus EBE, DNA binding assays based on electrophoretic mobility shift (EMS) measurements were performed in combination with double-stranded oligonucleotides encompassing the predicted binding sites. *AvrXa7* protein was produced in *Escherichia coli* and subjected to gel electrophoresis in the presence of ^{32}P -labeled double-stranded oligonucleotides derived from predicted binding sites of the wild-type candidate EBE for *AvrXa7* from the *Os-11N3* promoter (*Os-11N3oWT*), a mutant version (*Os-11N3oM2*), and the candidate EBE for *PthXo1* from *Os-8N3* (*Os-8N3oWT*) (Figure 8A). *AvrXa7* preferentially show greater retardation of labeled *Os-11N3oWT* in comparison to *Os-8N3oWT* (Figure 8B). Furthermore, the binding of the *Os-11N3oWT* could be competed with unlabeled *Os-11N3oWT*, but binding was not competitive with excess of the variant oligonucleotide *Os-11N3oM2* (Figure 8C).

Chromatin immunoprecipitation (ChIP) assays were performed with *AvrXa7* to determine if *AvrXa7* is associated in vivo with the respective promoter region of *Os-11N3*. Double FLAG-tagged genes for *AvrXa7* (*AvrXa7-2F*) and *PthXo1* (*PthXo1-2F*) were constructed and introduced into ME2, and rice leaves were inoculated with the respective strains. Prior to the ChIP analysis, FLAG-tagged versions of both *avrXa7* and *pthXo1* were found positive for expression by immunoblot analysis, the ability to induce the respective S genes, and TAL effector-specific virulence (Figures 9A to 9C). Rice chromatin complexes were retrieved by immunoprecipitation using anti-FLAG antibody and subjected to qPCR analyses using two respective sets of primers: one set for the promoter region (Figure 9D, *Os-11N3p*), including the predicted DNA binding elements; and a set for downstream untranslated sequences of *Os-11N3* (*Os-11N3-3'*). A primer set was included for the promoter region and predicted *PthXo1* binding site from *Os-8N3* as a control (Figure 9D, *Os-8N3p*). Enrichment of *Os-11N3p* was greatest in leaf samples prepared with *AvrXa7-2F* and FLAG antibody (Figures 9D, *Os-11N3p*). The promoter sequences of *Os-11N3* showed an ~ 7 -fold increase over the same DNA in anti-IgG complexes. Little or no amplification was observed in the same samples for *Os-11N3-3'* (Figure 9D) or the promoter region of *Os-8N3* (Figure 9D, *Os-8N3p*). A similarly tagged version of *PthXo1* (*PthXo1-2F*) was also constructed, introduced into ME2, and used to prepare immunoprecipitated complexes with FLAG antibodies. Complexes with *PthXo1-2F* were not enriched for *Os-11N3p*, indicating specificity of the *AvrXa7-2F/Os-11N3p* interaction (Figure 9D, *Os-11N3p/PthXo1-2F*).

DISCUSSION

We demonstrated that strains of *X. oryzae* pv *oryzae* can defeat the recessive resistance of *xa13* by the deployment of any one of the alternate major type III TAL effectors *PthXo2*, *PthXo3*, or *AvrXa7*. Furthermore, the ability of *PthXo3* and *AvrXa7* to defeat *xa13* is shown to be specifically due to the induction of the alternate S gene *Os-11N3*, a member of the *N3* gene family.

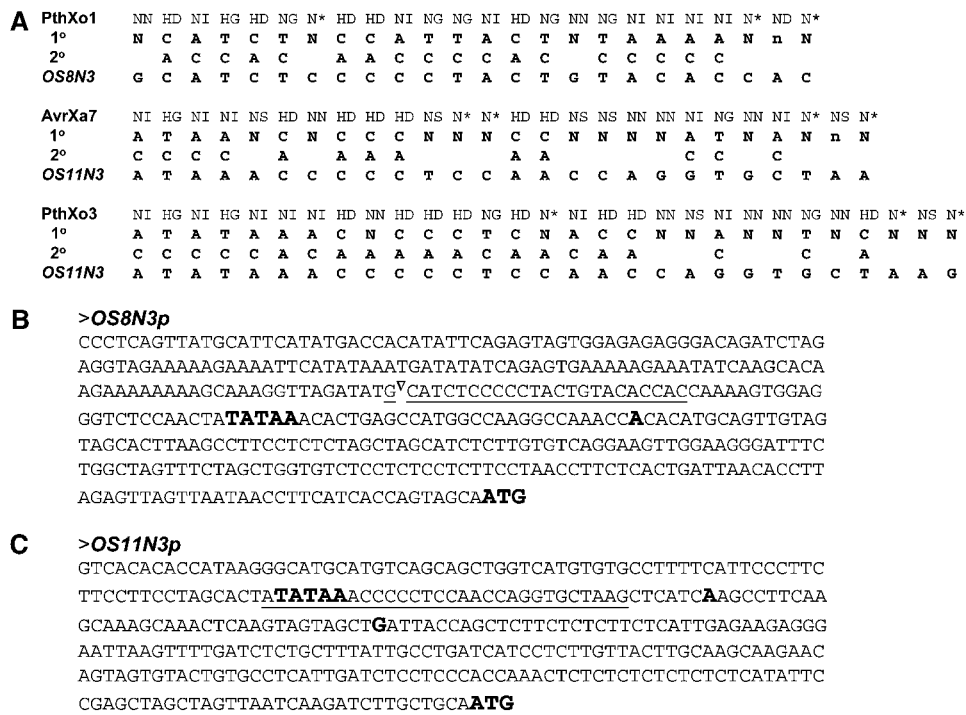


Figure 6. Candidate Effector Binding Elements in the Promoters of *Os-8N3* and *Os-11N3*.

(A) The predicted effector binding element of PthXo1, AvrXa7, and PthXo3 aligned with the corresponding two amino acid variables in the respective repeat region. 1° and 2° denote primary and secondary possible nucleotides as specified by the two amino acid variable residues of the respective repeats. Consensus nucleotides are indicated by the single letter code: N, A, C, G, or T. n, unassigned.

(B) The promoter region of *Os-8N3* (−397 to +3) from cultivar Nipponbare is shown. Predicted PthXo1 binding element is underlined, and the site of insertion in IRBB13 is indicated by a triangle next to the first nucleotide of the EBE. The start site for normal transcription A is indicated in large bold font immediately downstream of the TATA box.

(C) *Os-11N3* promoter sequence (−336 to +3) from cultivar Nipponbare with AvrXa7 and PthXo3 binding elements underlined. The start sites for normal transcription (A) and the alternate transcription in the presence of AvrXa7 (G) are indicated in large bold font.

Similar to previous results with *Os-8N3*, interference with *Os-11N3* expression during infection, either due to T-DNA insertion or RNA-mediated silencing, provided resistance against strains of the pathogen that rely solely on AvrXa7 or PthXo3 as the major TAL effectors for virulence. The circumvention of *xa13*-mediated resistance by AvrXa7 and PthXo3 involved the wholesale change in gene targets, in this case, the switch from *Os-8N3* to *Os-11N3*. Nevertheless, the actual basis of the switch, at least as demonstrated for AvrXa7, is the change in DNA sequence recognition as mediated by the repetitive regions of the two effectors. Although DNA binding was not measured specifically, we hypothesize that PthXo3 interacts specifically with the predicted PthXo3 binding site in the *Os-11N3* promoter. Compatibility, in the case of all three alternate major TAL effectors, did not entail the induction of *Os-8N3*. However, existence of PthXo3 illustrates a class of TAL effectors that arise due to recognition of variant sequences within the same promoter. PthXo3 is hypothesized to have arisen as an adaptation to evade *Xa7*-mediated resistance (Yang et al., 2005). The relatively small differences in the EBEs for PthXo3 and AvrXa7 permit induction of *Os-11N3*, while avoiding elicitation of *Xa7*, which, based on previously demonstrated requirements of the TAL effector transcription activation properties, is hypothe-

sized to require induction similar to *Xa27* and *Bs3* (Yang et al., 2000; Gu et al., 2005; Römer et al., 2007). Evidence for this hypothesis awaits further analysis of the binding specificities of AvrXa7 and PthXo3 as well as the characterization of *Xa7*.

The use of *Os-11N3* by *X. oryzae* pv *oryzae* also illustrates the dilemma faced by host plants. Simple inactivation of *Os-11N3* or *Os-8N3* is not an option for achieving resistance, since complete loss, in the case of *Os-11N3*, resulted in pleiomorphic and severe consequences for the plant, presumably due to the normal function in plant development. Homozygous plants for the *Os-11N3* insertion were stunted in several aspects of their development. The most conspicuous phenotype is delayed growth. No T-DNA mutants were available for the *Os-8N3* locus. However, silencing of *Os-8N3* resulted in plants with poor fertility. We are unaware of rice germplasm with recessive mutations for *Os-11N3* similar to *xa13* alleles of *Os-8N3*. Nonetheless, the finding that base substitutions within the AvrXa7 EBE disrupt effector function in the transient assays provides evidence that it may be possible to incorporate recessive mutations into the *Os-11N3* promoter using a variety of approaches. Recessive resistance might have advantages if it provided protection against both AvrXa7- and PthXo3-mediated virulence and did not interfere

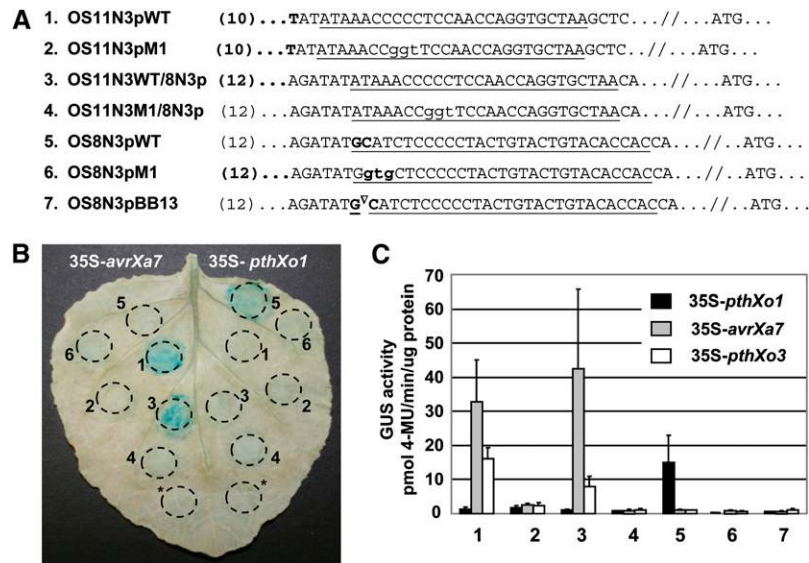


Figure 7. AvrXa7 Drives TAL Effector-Specific Host Gene Induction.

(A) Promoter fragments used in the transient expression assay. The consensus effector binding elements are underlined. The nonconsensus changes introduced in the mutant versions are in lowercase letters. Os-8N3pBB13 contains the insertion/deletion of *xa13* allele in IRBB13, and sequence is not shown. The first 10 (Os-11N3) or 12 (Os-8N3) bases upstream of the EBE and between the EBE region and the ATG are not shown. Each fragment was fused to the ATG of the *uidA* coding sequence.

(B) GUS assay on *N. benthamiana* using 35S-*avrXa7* (left sites) or 35S-*ptHXo1* (right sites). Sites are stained with X-gluc. Coinoculations with 35S-*ptHXo3* and combinations with promoter fragment Os-8N3pBB13 (7) are not shown. The T-DNA vector containing 35S-*avrXa7* or 35S-*ptHXo1* alone was infiltrated at sites indicated with an asterisk.

(C) Average GUS activity was calculated from triplicate coinoculations of the promoter fragments in **(A)** with 35S-*ptHXo1*, 35S-*avrXa7*, or 35S-*ptHXo3*. The numbers for each treatment with the indicated plant-expressed effector corresponds to the promoter fragments in **(A)**. Average GUS activity was measured from three excised leaf disks with hybrid promoters as indicated in **(A)** coinfiltrated with 35S-*ptHXo1* (black columns), 35S-*avrXa7* (gray columns), or 35S-*ptHXo3* (white). Activity on MUG substrate is expressed as pmol of 4-methylumbelliferone (4-MU) per μg of protein.

significantly with normal Os-11N3 expression. The fact that growth aberrancies were not observed in Os-11N3-silenced plants, possibly due to leaky expression of Os-11N3 in comparison to the T-DNA insertion line, also indicates that some change in Os-11N3 expression levels are probably not severely detrimental to plant growth and development.

As variants of the prototype TAL effector AvrBs3, the TAL effectors AvrXa7, PthXo1, PthXo2, and PthXo3 are predicted to bind specifically to host DNA elements that are defined by the sequence of the central repeats (Boch et al., 2009). AvrXa7, PthXo1, PthXo2, and PthXo3 each have unique repetitive regions, and, based on the prediction of the alignments of the AvrBs3 repeats and the cognate consensus target DNA element, each has a unique predicted binding site within the respective promoter regions (Boch et al., 2009; Moscou and Bogdanove, 2009). The results with AvrXa7 and PthXo1 corroborate the EBE model, and multiple approaches corroborate the link between AvrXa7, in particular, the EBE in the Os-11N3 promoter, and the induction of Os-11N3. ChIP analysis of AvrXa7-associated complexes yielded enrichment for the Os-11N3 promoter in comparison to sequences distal to the 3'-UTR region of Os-11N3, the promoter region of Os-8N3, or complexes retrieved by nonspecific IgG or PthXo1-2F. Additionally, AvrXa7 protein showed preferential retardation of a short oligoduplex representing the EBE in the

EMS analysis. Furthermore, the effector-specific induction of the reporter gene was observed in the heterologous *N. benthamiana* expression system, and mutations within the EBE for either PthXo1 or AvrXa7 abolished reporter gene induction, and replacement of the PthXo1-specific element with that for AvrXa7 resulted in AvrXa7-dependent gene induction. Although not examined in detail here, the results also indicated that the *xa13* allele of IRBB13 indeed had an effect on Os-8N3 expression in the *N. benthamiana* assay, supporting the hypothesis that the promoter polymorphisms within the predicted EBE for PthXo1 are the cause of the resistance phenotype in *xa13* plants. The predicted EBEs of Os-8N3 and Os-11N3 promoter regions were also recently and independently demonstrated to direct reporter gene expression in *N. benthamiana* (Römer et al., 2010). Detailed analyses of the Os-11N3 and Os-8N3 promoter sequences in relation to TAL effector-mediated expression should add more insight to TAL effector binding properties.

A limited number of recessive resistance genes have been characterized in various plant species, and fewer have demonstrated involvement in effector-mediated susceptibility. One previous case that involves type III effector-dependent recessive resistance is the *Arabidopsis* *RRS1-R* gene, which requires the Pop2 type III effector from *Ralstonia solanacearum* (Deslandes et al., 2002). However, the recessive nature of *RRS1-R* has yet to

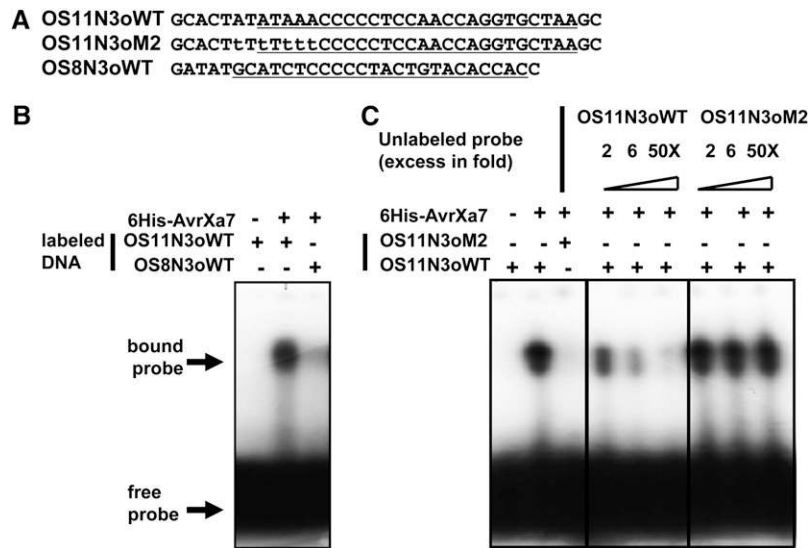


Figure 8. AvrXa7 Interacts with the Os-11N3 Promoter.

(A) Oligonucleotides used in EMS assays. The predicted effector binding elements within the respective promoters are underlined. The nucleotide changes in the mutant version Os-11N3M2 are in lowercase.

(B) The oligonucleotides for EMSA (shown in **[A]**) were incubated with 6His-AvrXa7. “+” or “-” indicates the presence or absence of the AvrXa7 or the oligonucleotides included in the reaction for each lane.

(C) Competition assay with increasing amounts of unlabeled Os-11N3WT or Os-11N3M2.

be explained, and the function of Pop2, either for virulence or avirulence, has not been deduced (Deslandes et al., 2003). It also should be noted that a susceptibility function for *RRS1-S*, the susceptible and dominant allele of *RRS1-R*, has not been identified. Recent progress in the analysis of effector-mediated virulence in two fungal diseases of wheat has also revealed evidence for gene-for-gene susceptibility. Both the tan spot pathogen *Pyrenophora tritici-repentis* and the stagonospora nodorum blotch pathogen *Stagonospora nodorum* are dependent on a family of effector (toxin) genes whose virulence effect is dependent on a set of corresponding host susceptibility genes (Chu et al., 2010). The recessive resistance to specific fungal races is due to the lack of the cognate susceptibility gene. Two additional recessive naturally occurring resistance genes have been cloned. *mlo* provides resistance against all races of powdery mildew of barley, while the *xa5* gene from rice provides resistance against a broad range of strains of *X. oryzae* pv *oryzae* (Iyer and McCouch, 2004; Piffanelli et al., 2004; Jiang et al., 2006). Neither gene has been demonstrated to interfere with effector-mediated susceptibility. *xa5* encodes a single amino acid residue variant of the transcription factor TFIIA- γ 5 and, as part of the preinitiation complex of eukaryotic transcription, has potential to function in altering TAL effector function; evidence has been presented that *xa5* can interfere with TAL effector-mediated resistance (Gu et al., 2009).

Bacterial blight disease of rice is proving to be an excellent system for the study of coadaptation of host and bacterial pathogen. On the pathogen side, the evidence indicates that repeat shuffling among the TAL effector genes and the targeting of new DNA elements within the S gene promoters has provided

the pathogen with a mechanism to circumvent resistance and promote virulence. The TAL effector family of genes is unusual in the large number of variant genes within strains and in different pathovars and species, perhaps owing to their mechanism of action. Strains of *X. oryzae* pv *oryzae* have high copy numbers of the genes within their genomes, and the presence of multiple genes with their corresponding repetitive regions may facilitate the appearance of TAL effectors through homologous recombination. It is also interesting to note that AvrBs3, AvrXa7, and AvrXa27 bind EBEs that either have a TATA-like sequence within the element, and the predicted EBEs for PthXo3, as shown here, also encompasses the sequence (Boch et al., 2009; Römer et al., 2009). PthXo1 alone targets a non-TATA element. The effectors have been proposed to potentially compete for the TATA binding protein. Alternatively, the occurrence of the motif in EBEs may reflect the presence of the consensus with many host promoters, reducing the complexity of the target for newly evolved effector genes. Targeting TATA motifs may also reflect pathogen adaptation to host sequences that are more difficult to change.

On the host side, the fact that two major S genes of rice for bacterial blight are all closely related members of the *N3* family points to an intrinsic property of the proteins conferring the ability of the bacterium to grow within the host. All strains of *X. oryzae* pv *oryzae* may require induction of at least one member of the *N3* gene family for virulence. Os-11N3 does not appear to be a recent duplication or the result of selection for a pathogen resistant replacement for Os-8N3. Phylogenetic analysis indicates that Os-11N3 is a member of a distinct clade of *N3* genes, possibly serving similar function to Os-8N3 in a different developmental context. Alterations in Os-11N3 expression resulted in

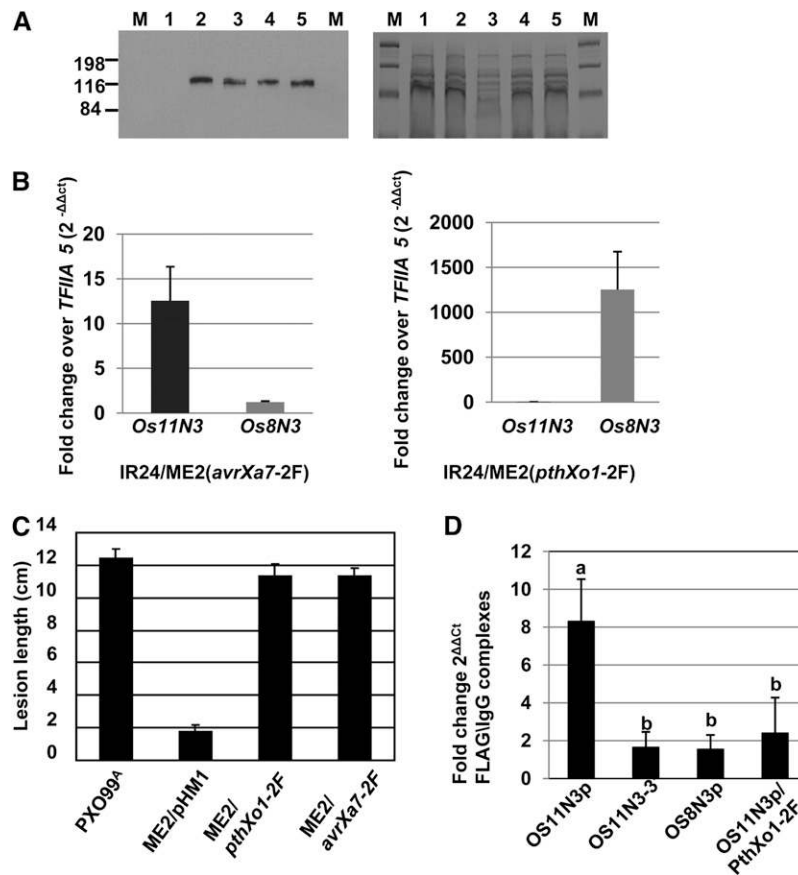


Figure 9. AvrXa7 Interacts with the Os-11N3 Promoter in Rice.

(A) Immunoblot analysis of AvrXa7-2F and PthXo1-2F protein expression, inferred from M2 FLAG monoclonal antibody recognition of internal double FLAG epitopes in bacterial strains carrying FLAG-tagged AvrXa7-2F and PthXo1-2F. Analysis of protein from two colonies is shown. Coomassie blue-stained gel is shown at right. M, protein molecular size standards. Numbers at left indicate kilodaltons. Lanes: ME2 (pHM1); 2, ME2(avrXa7-2F-1); 3, ME2(avrXa7-2F-2); 4, ME2(pthXo1-2F-1); 5, ME2(pthXo1-2F-2).

(B) FLAG-tagged versions of AvrXa7 and PthXo1 induce the respective S gene when produced in ME2. Induction was measured in $2^{\Delta\Delta Ct}$. Three 14-d-old rice seedlings were inoculated with the indicated strain, and total RNA was isolated from three leaves and subjected to qRT-PCR.

(C) *avrXa7-2F* and *pthXo1-2F* confer virulence on ME2. Four-week-old rice plants were inoculated with the respective strains (indicated below each column) by leaf tip clipping inoculation. Lesion lengths were measured 12 d after inoculation on 10 inoculated leaves for each treatment. Error bars indicate 1 SD.

(D) qPCR analysis of AvrXa7-2F (first three columns) and PthXo1-2F (fourth column) immunoprecipitated complexes from leaf infection sites using primers for the indicated DNA fragment. Fold changes in average cycle numbers were compared with average cycle numbers of the same PCR products in complexes immunoprecipitated with IgG control antibodies. The values are the averages of three independent leaf inoculations with the exception of the fourth column, which is the average of two inoculations. Values that do not differ significantly at $P < 0.05$ level are indicated by the same lowercase letter. Significance was determined using ANOVA and the Tukey HSD test (F-statistic, 13.68, $P = 0.0026$). Error bars indicate 1 SD.

different developmental phenotypes in comparison to Os-8N3. Previous studies indicated that Os-8N3 is upregulated during pollen development, which is consistent with the observation of reduced fertility in silenced plants (Chu et al., 2006; Yang et al., 2006). Although not analyzed here, published microarray expression data reveal that Os-11N3 is upregulated in root tissues, indicating a primary function in roots (Li et al., 2009). The fact that some apparent full-length 5'-RACE cDNA clones were obtained in this study from leaf tissue may indicate some low level expression in multiple tissue types. The cognate S gene was not identified for PthXo2, and host gene expression studies for

PthXo2 are in progress. It is interesting to note that *UPA16*, a bell pepper gene upregulated by AvrBs3, is also a member of the N3 gene family (Kay et al., 2009). N3 relatives are found throughout the plant kingdom as well as mammals, arthropods, and nematodes (Yang et al., 2006). Recent evidence has revealed two possible functions for N3 proteins in plants and animals. One report indicates that Os-8N3 interacts with copper transport proteins of rice, functioning to redistribute copper and reduce the copper ion concentration within the xylem. The authors propose that normal copper levels in the xylem are inhibitory to bacterial growth, and the reduction in level facilitates bacterial growth

within the host (Yuan et al., 2010). Another report indicates that N3 proteins, including Os-8N3 and Os-11N3, can function as low-affinity glucose transporters, allowing ingress or efflux of glucose into or out of cells according to the glucose concentration gradient. In the latter model, the pathogen induces the host to release glucose into the apoplastic and xylem fluids, stimulating pathogen growth and virulence (Chen et al., 2010). Further experimentation will be required to embellish either of these new models, although they are not necessarily mutually exclusive.

METHODS

Plant Material, Plasmids, and Bacterial Strains

Rice (*Oryza sativa*) varieties IR24, IRBB13, Nipponbare, Hwayoung, and Kitake were used in the study. Line PFG_3D-03008 was derived from Hwayoung (Jeong et al., 2006). Seeds of rice variety Nipponbare (accession number PI 514663) were provided by the USDA-Agricultural Research Service National Small Grains Collection. IR24 and IRBB13 seeds were obtained from the International Rice Research Institute (courtesy of Casiana Vera Cruz). Kitake seeds were provided by Pamela Ronald (University of California, Davis). Seeds of the T-DNA insertion line PFG_3D-03008 and its parental strain Hwayoung were provided by the POSTECH Biotech Center in Pohang University of Science and Technology. All rice plants were grown in growth chambers with temperature of 28°C, relative humidity of 85%, and photoperiod of 12 h. *Xanthomonas oryzae* pv *oryzae* strains and plasmids are listed in Supplemental Table 1 online.

Expression Analyses

The rice leaves were inoculated with indicated bacterial strains and used for total RNA extraction at indicated time points as described in the text. RNA was extracted using the TRI reagent from Ambion, and RNA concentration and quality were measured using an ND-1000 Nanodrop spectrophotometer (Nanodrop Technologies). Fifteen micrograms of total RNA for each sample were separated in 1% agarose gel and blotted on Hybond N⁺ membrane (Amersham Pharmacia). The blot hybridization was performed with specific probes as indicated in the text at 65°C with appropriate buffer. The probe for Os-8N3 was prepared from cDNA using the primer set of RT-8N3-F and 8N3Probe-R. RT-PCR was performed on RNA extracted from leaves inoculated with bacteria as indicated in the text. Total RNA was extracted 24 h after inoculation, and 1 μg of RNA from each inoculation was treated individually with amplification grade DNase1 (Invitrogen) followed by cDNA synthesis using the iScript Select cDNA synthesis kit (Bio-Rad). Primers 11N3RNAi-F and 11N3RNAi-R were used for Figure 2A, and primers RT-TF2-5F and RT-TF2-5R were used to PCR amplify *TFIIAγ5*. Primer sequences are provided in Supplemental Table 2 online. qPCR and qRT-PCR were performed on DNA or RNA extracted from leaves 24 h after inoculation, respectively. For qRT-PCR, 1 μg of total RNA was subjected to DNase I (Invitrogen) treatment to eliminate the genomic DNA contamination and then to first-strand cDNA synthesis using the iScript cDNA Synthesis kit (Bio-Rad). cDNA derived from 25 ng of total RNA was used for each real-time PCR, which was performed on Stratagene's Mx4000 multiplex quantitative PCR system using the iQ SYBR green Supermix kit (Bio-Rad). The gene-specific primer sequences are provided in Supplemental Table 2 online. The average threshold cycle (Ct) was used to determine the fold change of gene expression. *TFIIAγ5* expression was used as an internal control. The 2^{-ΔΔCt} method was used for relative quantification (Livak and Schmittgen, 2001).

Phylogenetic Analysis

Alignment and phylogenetic analyses were conducted using ClustalW (Thompson et al., 1994) and MEGA version 4 for unrooted phylogenetic tree construction using the minimum evolution method (Tamura et al., 2007). The tree is depicted in rooted format using the midpoint between each node. Alignments are provided in Supplemental Figure 1 and Supplemental Data Set 1 online. Bootstrap support value for 1000 reiterations is indicated above each node.

Genotyping of T-DNA Line PFG_3D-03008

DNA was extracted from a single leaf of each progeny plant and genotyped with the following primers: Os11g-F (wild-type locus forward primer) and Os11g-R (wild-type locus reverse primer); and 2772 RB-F (pGA2772 right border T-DNA primer). Primer sequences are provided in Supplemental Table 2 online.

Rice Transformation and Gene Construction

For construction of Os-11N3 RNAi plants, a 341-bp fragment specific to Os-11N3 was PCR amplified with primers 11N3RNAi-F and 11N3RNAi-R. The product was cloned into pTOPO/D-ENTR vector, sequenced, and recombined into pANDA (Miki and Shimamoto, 2004) through LR recombinase according to the instructions of the manufacturer (Invitrogen). The construct was transformed into *Agrobacterium tumefaciens* strain EHA105. Calli from immature embryos of rice cultivar Kitake were initiated and transformed using *Agrobacterium* as described (Hiei et al., 1997).

Virulence Assays

The fully expanded rice leaves at the stages indicated in the text were inoculated by leaf tip clipping with scissors that were immersed in bacterial suspensions of optical density of 0.5 at 600 nm ($\approx 5.0 \times 10^7$ cell forming units per mL) immediately prior to each clipping as described (Kauffman et al., 1973). Symptoms were scored by measuring lesion length. Significance between treatments as assessed on the basis of a P value of <0.05 using the Tukey test after analysis of variance (ANOVA).

5'-RACE cDNA Analysis

The 5'-RACE cDNAs were derived from leaf tissue of cultivar Nipponbare 24 h after inoculation with ME2 or ME2(*avrXa7*). RNA was extracted using the TRI reagent (Ambion) and subjected to 5'-RACE RT-PCR analysis using the primer 5'-CTTGCTTGCAAGTAACAAGAG-3' in place of a poly-dT primer and the SMARTer RACE cDNA amplification kit (Clontech). Individual cDNAs were cloned in pCR2.1 using the TOPO cloning kit (Invitrogen) and sequenced.

Transient Expression Assays

Promoter-*GUS* constructs were made by amplifying the promoter regions using specific primers given below, and amplicons obtained were digested with *HindIII* and *XbaI* and cloned into *HindIII* and *XbaI* sites in pBI121 by replacing the 35S promoter (Jefferson et al., 1987). The specific primers (sequences provided in Supplemental Table 2 online) for each promoter construct are as follows: OS8N3pWT (8pG-F and 8pG-R), OS8N3pM (8pMGF and 8pG-R), OS8N3pBB13 (BB13F and 8pG-R), OS11N3pWT (11pG-F and 11pG-R), OS11N3pM (11pMGF and 11pG-R), OS11N3WT-OS8N3p' (11-8PG-F and 8pG-R), and OS11N3M-OS8N3p' (11M-8pG-F and 8pG-R). All constructs were sequenced before introducing into *Agrobacterium*. For each assay, *Agrobacterium* transformants with various constructs were streaked on Luria-Bertani (LB) agar supplemented with kanamycin (50 μg/mL) and rifampicin (15 μg/mL)

antibiotics and grown at 28°C for 2 d. A single colony was inoculated in 5 mL liquid LB media supplemented with kanamycin (50 µg/mL) and rifampicin (15 µg/mL), 1 mL of the overnight culture was subcultured in 50 mL liquid LB supplemented with kanamycin (50 µg/mL) to an OD₆₀₀ of 0.6. The bacterial cells were then collected by centrifugation at 4°C for 10 min at 3000 rpm. The cells from each centrifugation were resuspended in 50 mL *Agrobacterium* inoculation buffer (4.8 gm MES, 5 mL 1 M MgCl₂, and 0.147 g acetosyringone in 500 mL water, pH 5.6) and activated at 28°C for 3 h. Coinoculation was done by mixing the cultures in 1:1 ratio prior to inoculation. Bacterial suspension (100 µL) was infiltrated into the leaf at each inoculation site. The inoculation was done on fully opened leaves (three leaves per treatment), and the leaves were harvested 40 h after inoculation and incubated at 37°C in GUS reagent (100 mM phosphate buffer with 0.5% Triton X-100, 10 mM EDTA, 0.5 mM each of X-gluc [5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexylammonium salt] and potassium ferri- and ferrocyanide) for 7 h and thereafter cleared using 70% ethanol (Jefferson et al., 1987). Average GUS activity was measured in triplicate from extracts of the inoculated portions of leaves. The tissue (~50 mg) was excised and homogenized in 1.5-mL micro-centrifuge tubes with 1 mL of extraction buffer (50 mM NaPO₄, pH 7.0, 1 mM Na₂EDTA, 10 mM DTT, 0.1% sodium lauryl sarcosine, and 0.1% Triton X-100. GUS activity was measured using the MUG substrate and expressed as pmol 4-methylumbelliferone/µg protein.

Promoter primers are provided in Supplemental Table 2 online.

EMS Assays

Six-His-tagged AvrXa7 protein was expressed and purified from *Escherichia coli* BL21 with Ni-NTA agarose (Qiagen). Protein concentration was determined using a Bradford reagent kit (Bio-Rad). Complementary oligonucleotides were annealed and 5' end labeled with [³²P]ATP catalyzed by T4 kinase. Labeled double-stranded DNA was mixed with AvrXa7 in a reaction containing Tris-HCl (15 mM, pH 7.5), KCl (60 mM), DTT (1 mM), glycerol (2.0%), MgCl₂ (2.5 mM), poly(dI.dC) (50 ng/µL), EDTA (0.2 mM), labeled DNA (50 fmol), unlabeled DNA (0 to 2.5 pmol), and AvrXa7 (350 fmol). The binding reactions were kept at room temperature for 30 min before being loaded on an 8% TBE polyacrylamide gel.

ChIP Assays

Versions of AvrXa7 and PthXo1 with two FLAG epitope coding sequences were constructed, and the genes for the effectors were introduced into strain ME2. For immunoblot analysis, *X. oryzae* pv *oryzae* grown in TSA media was adjusted to the same OD₆₀₀ = 0.5 using the same medium. Bacteria (150 µL) was boiled with the same amount of 2× SDS-PAGE loading buffer, and 20 µL was loaded onto a 10% SDS-PAGE gel. Proteins separated by SDS-PAGE were transferred to a polyvinylidene fluoride membrane and immunoblotted using monoclonal FLAG antibody (Sigma-Aldrich). The secondary antibodies (goat anti-mouse horseradish peroxidase-conjugated; Millipore) bound to the membrane were detected using ECL protein gel blotting reagents (Pierce, Thermo Scientific) and x-ray film (Kodak). Two-week-old rice plants were syringe-inoculated with ME2(avrXa7) or ME2(pthXo1) with the suspension of OD₆₀₀ = 0.5. S gene induction was performed as described above using gene-specific primers. Virulence assays were performed as described above. ChIP assays were performed as described with following modifications (Haring et al., 2007): 3.0 g of inoculated leaf material was harvested 20 h after inoculation, fixed using 1.5% formaldehyde. The tissue was sonicated using Virsonic 50 (output control 6) for 8 × 15 s. The protease inhibitor cocktail for plant cell and tissue extracts (Sigma-Aldrich; P9599) was used. Immunoprecipitation was done using monoclonal M2 FLAG antibody (12 µg/mL; Sigma-Aldrich). The mouse serum (12 µg/mL) was used as control. PCR on enriched DNA sample was done using the following primers: 11N3-5' (11N3- 5'F and 11N3-5'R), 11N3-3' (11N3-3'F and

11N3-3'R), 8N3-5' (8N3-5'F and 8N3-5'R), and 8N3- 3' (8N3-3'F and 8N3-3'R). Primer sequences are provided in Supplemental Table 2 online. Quantitative real-time PCR measurements used the average threshold cycle (Ct) to determine the fold change of DNA content. The 2^{ΔΔCt} method was used for relative quantification (Livak and Schmittgen, 2001).

Accession Numbers

Sequence data from this article can be found in the National Center for Biotechnology Information database under the following accession numbers: AK070510 (Os-8N3), AK101913 (Os-11N3), and NM_001060961 (Os-TFIIAγ5).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. ClustalW Alignment of N3 Proteins.

Supplemental Figure 2. Alignment of Os-011N3 Transcription Sites after Inoculation with ME2 or ME2 (*avrXa7*).

Supplemental Table 1. Strains and Plasmids Used in This Study.

Supplemental Table 2. Primers Used in This Study.

Supplemental Data Set 1. Text File of N3 Protein Alignments.

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Rice *xa13* Recessive Resistance to Bacterial Blight Is Defeated by Induction of the Disease Susceptibility Gene *Os-11N3*

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