Running title: Cloning of the *M. oryzae AVR-Pia* locus

Molecular cloning and characterization of the *AVR-Pia* locus from a Japanese field isolate of *Magnaporthe oryzae*

SHINSUKE MIKI,[1] KOTARO MATSUI,[1] HIDEKI KITO,[1,2] KEISUKE OTSUKA,[1] TAKETO ASHIZAWA,[3] NOBUKO YASUDA,[4] SATORU FUKIYA,[1] JUNKO SATO,[1] KAZUYUKI HIRAYAE,[3] YOSHIKATSU FUJITA,[4] TOSHIHIKO NAKAJIMA,[2] FUSAO TOMITA,[1] AND TERUO SONE[1 *]

[1]Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan; [2]National Agricultural Research Center for Tohoku Region, Daisen, Akita 014-0102, Japan; [3]Hokuriku Research Center, National Agricultural Research Center, Joetsu, Niigata 943-0193, Japan; [4]National Agricultural Research Center, 3-1-1 Kannondai, Tsukuba, Ibaraki 305-8666, Japan

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Correspondence: Tel.: +81 11 706 2502; Fax: +81 11 706 4961; E-mail: sonet@chem.agr.hokudai.ac.jp

SUMMARY

In order to clone and analyse the avirulence gene *AVR-Pia* from Japanese field isolates of *Magnaporthe oryzae*, a mutant of the *M. oryzae* strain Ina168 was isolated. This mutant, which was named Ina168m95-1, gained virulence towards the rice cultivar Aichi-asahi, which contains the resistance gene *Pia.* A DNA fragment (named PM01) that was deleted in the mutant and that co-segregated with avirulence towards Aichi-asahi was isolated. Three cosmid clones that included the regions that flanked PM01were isolated from a genomic DNA library. One of these clones (46F3) could complement the mutant phenotype, which indicated clearly that this clone contained the avirulence gene *AVR-Pia.* Clone 46F3 contained insertions of transposable elements. The 46F3 insert was divided into fragments I–VI, and these were cloned individually into a hygromycin-resistant vector for the transformation of the mutant Ina168m95-1. An inoculation assay of the transformants revealed that fragment V (3.5 kb) contained *AVR-Pia*. By deletion analysis of fragment V, *AVR-Pia* was localised to an 1199-bp DNA fragment, which included a 255-bp open reading frame with weak homology to a bacterial cytochrome-c-like protein. Restriction fragment length polymorphism analysis of this region revealed that this DNA sequence co-segregated with the *AVR-Pia* locus in a genetic map that was constructed using Chinese isolates.

INTRODUCTION

The ascomycete fungus *Magnaporthe oryzae* (Couch) is the causal agent of rice blast disease, which is the most devastating disease of rice plants globally. In order to control the disease, many strategies have been developed, but often such attempts are negated by mutations that occur in the genome of the pathogen. One approach is the usage of blast-resistant cultivars that carry major resistance (R) genes, which interact specifically with fungal avirulence (AVR) genes to trigger the hypersensitive reaction (HR). Mutations that affect the transcription and/or function of the products of AVR genes disrupt the resistance. Therefore, these genes are a suitable target for research that is aimed at improving the longevity of resistant cultivars.

The gene-for-gene hypothesis, which states that plants contain single dominant R genes that confer resistance to pathogens with a complementary AVR gene, was established by Flor (1971). It has been verified in many plant–pathogen systems, including the rice (*Oryza sativa*) – blast fungus (*M. oryzae*) interaction (Laugé and De Wit, 1998). Several AVR genes have been cloned from *M. oryzae*, and mutations in some of these genes have also been analysed. *AVR-Pita*, which corresponds to the rice R gene *Pita*, encodes a zinc-metalloprotease-like protein, and is located near a telomere. The main cause of mutations in this gene is the introduction of deletions due to the instability of the telomere (Orbach *et al.* 2000). In addition, when a DNA-type transposon *Pot3* is inserted into the promoter region of the gene, the fungus gains virulence towards a cultivar that contains the *Pita* gene (Kang *et al.* 2001). A recent study by Khang *et al.* (2008) has revealed that multiple *AVR-Pita* gene homologues are contained in the genome of several different isolates and form a multigene family, and thus the original *AVR-Pita* gene has been renamed *AVR-Pita1*. Another AVR gene *ACE1*, which confers avirulence towards the rice cultivar *Pi33*, encodes a polyketide synthase that is fused to a non-ribosomal protein synthetase

(PKS-NRPS). This protein is expected to participate in secondary metabolism. *ACE1* differs from other AVR genes because its enzymatic activity is required for the induction of *Pi33* resistance; it is probable that the secondary metabolism product acts as the elicitor (Böhnert *et al.* 2004). Insertion of a MINE retrotransposon into the ACE1 gene is responsible for the virulence of strain 2/0/3 (Fudal *et al*. 2005). *AVR1-CO39* has been cloned (Farman and Leong, 1998), but further details of the protein that is encoded by this gene are still unknown (Peyyala and Farman, 2006). Several types of deletion in this gene have been reported in field isolates that are virulent towards cultivar CO39 (Farman *et al*. 2002). *PWL2*, a host-species specificity gene, has been cloned, but the function of the protein that it encodes is also still unknown (Sweigard *et al.* 1995). Overall, these studies have characterised certain AVR genes in *M. oryzae*, and therefore, have contributed to our understanding of the molecular basis of gene-for-gene interactions, especially in the case of the *Pita*/*AVR-Pita* pair, which shows a direct protein–protein interaction (Jia *et al.* 2000).

To improve resistance, large numbers of R genes have been introduced into rice cultivars, and many of these are used in practical rice cultivation. In Japan, rice multilines, which are sets of near-isogenic lines contain different R genes, have been introduced and used successfully to control blast epidemics in cultivated rice (Koizumi *et al.*, 2004). For the sustainable use of the multilines, it is important to elucidate the rate at which, and the mechanisms by which, mutations arise in pathogen AVR genes that overcome resistance, in order to estimate the longevity of the R genes in the multilines (Koizumi *et al*., 2004). In addition, an increase in the number of cloned AVR/R gene pairs will permit the structural analysis of these interactions and allow the molecular design of compounds that trigger the R genes. Such compounds will be candidates for novel agrochemicals. At present, the number of cloned AVR genes does not match the number of

rice R genes that have been introduced into blast-resistant multilines in Japan and other countries.

Recent studies of bacterial plant pathogens have revealed that some AVR gene products act as effectors that suppress microbe-associated molecular pattern-triggered immunity (He *et al.* 2007). Such AVR gene products are called type III effectors, because they are secreted into host plant cells by the type III secretion system. These effector proteins contain a secretion signal sequence that is specific to this secretion system, and can be used as a molecular signature in screening for AVR protein candidates. Comparative genome and secretome analysis, which is based on recent progress in the genome sequencing projects of fungi and oomycetes, is an effective way to screen for effector proteins that correspond to AVR gene products, in addition to other pathogenicity-related proteins (Soanes *et al*. 2007, 2008). In particular, the RXLR-EER motif, which is found in the effector proteins of oomycete plant pathogens, is thought to correspond to the secretion signal sequence. In filamentous ascomycetes, however, no such signal sequences have been identified, though some Pfam domains that are specific to the secretome of plant pathogens have been found.

AVR-Pia, the AVR gene in *M. oryzae* that triggers the HR in rice cultivars with the *Pia* R gene, was selected as the target in the present study. *Pia* was found originally in the Japanese rice cultivar Aichi-asahi, which is one of the Japanese cultivars that are differential for blast race, and has been introduced subsequently into many Japanese cultivars, including multilines (Yamasaki and Kiyosawa 1966; Koizumi *et al*., 2004). In order to clone AVR genes, positional cloning strategies are often used because reverse genetic approaches are not effective. This is presumably caused by the lack of structural similarity that has been reported among cloned AVR genes (Sweigard *et al.* 1995; Farman and Leong 1998; Orbach *et al*. 2000; and Böhnert *et al.*

2004). For positional cloning, a genetic map of sufficient accuracy to allow chromosomal walking is essential. A genetic map has been developed using the Japanese rice-pathogenic isolate Ina168 and the hermaphrodite strain Guy11, but it is not accurate enough for chromosomal walking (Fukiya *et al.*, 2001). More recently, chromosomal mapping of *AVR-Pia* has been achieved using hermaphrodite strains of *M. oryzae* that have been isolated in China (Yasuda *et al*. 2006; Chen *et al*., 2007).

The cloning of AVR genes has often been assisted by the use of AVR mutants. Sweigard *et al*. (1995) and Orbach *et al.* (2000) have used such mutants to identify AVR genes in a cosmid clone and telomere region, respectively. Such spontaneous mutants, in which host cultivar specificity has been affected, may contribute to the cloning of AVR genes in field isolates with low fertility. However, it is necessary to identify the causative mutation, because the possibility of multiple mutations in a particular mutant cannot be eliminated.

In order to clone AVR genes from Japanese isolates of *M. oryzae*, cross 5307 has been established by crossing the hermaphrodite strain Guy11 with progeny 2107-33, which were derived from a cross between Guy11 and Ina168. However, a model mapping experiment that has examined the kasugamycin-resistance locus in cross 5307 has resulted in a map that, as stated above, is unsuitable for map-based cloning (Fukiya *et al.* 2001). In the present study, we utilized cross 5307 to analyse the linkage of a DNA fragment that contained a mutation that occurred in a host cultivar specificity mutant of Ina168. This mutation was identified by random amplified polymorphic DNA (RAPD) analysis of Ina168 and its mutant. If the DNA fragment that contained the mutation showed strong linkage with avirulence towards the rice cultivar in which the mutant strain was pathogenic, then the DNA fragment might contain, or be tightly linked with, the AVR gene. The mutation in the DNA fragment was proposed to cause the loss of

function of the AVR gene, and therefore, a cosmid clone that contained this DNA fragment should also contain the AVR gene. The design of the present study was based on this hypothesis, and identified a 1199-bp DNA region that contained *AVR-Pia*.

RESULTS

Isolation of the mutant Ina168m95-1

Strain Ina168, which is incompatible with Aichi-asahi, was used as the source of *AVR-Pia*, because the strain was used originally for the identification of the *Pia* R gene (Yamasaki and Kiyosawa 1966). In addition, *AVR-Pia* was expected to be segregated in cross 5307, because 2107-33 was incompatible with Aichi-asahi (= *AVR-Pia*) but Guy 11 was compatible (= *avr-Pia*) (Table 1; Fukiya *et al*. 2001). Cross 5307 showed low fertility and thus was not suitable for developing a genetic map (Fukiya *et al*. 2001). However, it was regarded as useful in this study for checking the linkage between the mutation of interest and avirulence towards Aichi-asahi.

The spontaneous host-cultivar specificity mutant of Ina168 was isolated from a compatible lesion that occurred on a leaf of Aichi-asahi that had been inoculated with Ina168 conidia. The mutant, which was named Ina168m95-1, showed a change in host-cultivar specificity only towards *Pia* among the Japanese differential cultivar sets (Yamada *et al*. 1976). Other physiological features, such as sporulation of the mutant strain, were indistinguishable from those of the parental strain, Ina168 (data not shown).

RAPD analysis and the PM01 fragment

Strain Ina168 and the mutant Ina168m95-1 were screened by RAPD analysis to identify the DNA fragment that contained the mutation (Williams *et al*. 1990). Among the 240 random 10-mer primers tested, one primer, OPM-01 (5’-GTTGGTGGCT-3’), produced a polymorphic

DNA band (designated PM01) of 1.2 kb that was amplified from Ina168 but not from the mutant (Fig. 1A). PM01 was cloned into a plasmid vector and used as a probe for Southern analysis of chromosomal DNA from the two strains. Hybridization was observed only with the parental strain; thus, the PM01 fragment was deleted in the mutant (Fig. 1B).

Analysis of segregation of avirulence towards Aichi-asahi and of the PM01 RFLP among the progeny of cross 5307

Twenty-two progeny of cross 5307 were tested for their pathogenicity toward Aichi-asahi. As shown in Fig. 2, the pathogenicity segregated 9:12, which fits a 1:1 ratio in a χ^2 test of goodness-of-fit ($\chi^2 = 0.43$, $P = 0.51$), and shows that *AVR-Pia* is a single gene. The pathogenicity of one progeny, 5307-20, could not be determined. This strain had lost virulence towards Fujisaka 5, which is a cultivar that is susceptible to both 2107-33 and Guy11 (Fukiya *et al*. 2001), and thus it might have lost its general pathogenicity towards rice. All other progeny showed virulence towards Fujisaka 5. Genomic DNA from the parents and progeny of cross 5307 was digested with *Bam*HI and used for restriction fragment length polymorphism (RFLP) analysis of the PM01 fragment (Fig. 2). PM01 was detected as a 1.8-kb band in 2107-33, the *AVR-Pia* parent, but was not detected in Guy11. The PM01 RFLP and avirulence toward Aichi-asahi co-segregated perfectly, except in the case of 5307-20. This result suggests strongly that deletion of the PM01 DNA fragment was likely to correspond to the mutation that occurred in the *AVR-Pia* gene of Ina168m95-1.

Identification of a cosmid clone that complements *AVR-Pia*

We have constructed previously a library of Ina168 genomic DNA, using the cosmid vector pMOcosX (Orbach 1994), that covered the genomic DNA length five times and consisted of 4800 clones (Kito *et al*. 2003). This library was screened for positive clones. Among these,

three clones, 7B2, 9E12 and 46F3, were selected by PCR-sib selection and colony hybridisation as containing the PM01 DNA fragment. These three cosmid clones were then used to transform Ina168m95-1. A pathogenicity assay of these transformants revealed that clone 46F3 contained the *AVR-Pia* gene. Three out of eight 46F3 transformants showed complementation of avirulence towards Aichi-asahi, whereas none of the transformants of the other two cosmid clones showed any complementation (Table 2). The lack of complementation in five of the 46F3 transformants might have been caused by incomplete integration of the cosmid insert into the fungal genome.

The nucleotide sequence of clone 46F3 was determined by a shotgun method (Fig. 3). The insert of the clone was 38,604 bp in length, and the PM01 DNA fragment was located in the 5′ region of the sequence. BLAST searches (Supporting Information Fig. S1) revealed the insertion of the transposons *Occan* (Kito *et al.* 2003) and *Pot3-a,b* (which share 80% homology with *Pot3*), and clusters of repetitive, transposon-related sequences, *Pot2* (Kachroo *et al.* 1994), *Pot3* (Farman *et al*. 1996a), and the non-long terminal repeat (LTR) retrotransposon MGL (Meyn *et al*. 2000), together with solo LTRs of Pyret (Nakayashiki *et al*. 2001), MGLR-3 (Kang, 2001), RETRO 6, and RETRO 7 (Khang *et al.* 2008). Some other portions of the insert showed homology with genome sequence data (Dean *et al*. 2005). However, these regions were not contiguous in the genomic sequence, and included sequences from supercontigs 5.193 (chromosome 7), 5.195 (chromosome 1), and 5.189 (chromosome 5). The DNA region that was homologous with supercontig 5.195 also showed similarity with contig 5.193, but the similarity was lower than that with 5.195 (BLAST score for the alignment with supercontig 5.195 was 4286, whereas that with supercontig 5.193 was 876). These genome-homologous DNA regions were interrupted by the inserted transposons or clusters of transposon-like repetitive sequences. Other parts of the cosmid sequence did not show significant homology with the genomic

sequence data. The sequence was deposited in the DDBJ/EMBL/Genbank database under accession number AB434708.

Only the transformant that carried fragment V showed avirulence to Aichi-asahi

In order to determine the location of the *AVR-Pia* gene in cosmid clone 46F3, the insert was subcloned and complementation tests were performed using the subclones. Transposons or clusters of repetitive elements in the 46F3 insert were assumed not to be AVR genes. Therefore the insert was divided into six fragments, I–VI, that did not contain these sequences (Fig. 3). These fragments were amplified by PCR using the combinations of primers listed in Supporting Information Tables S1 and S2, and subcloned into the vector pCSN43-DEST. The subclones that contained each of the six fragments, pCSN43-DEST-LR-I to VI, were digested with *Sac*I and introduced into strain Ina168m95-1 by transformation. Out of the 81 transformants obtained, 20 were selected through Southern analysis: three transformants for each of fragments I–IV and VI, and five transformants for fragment V. The virulence of these transformants towards Aichi-asahi (*Pia*) was assessed by spray inoculation (Table 2). All 15 transformants that contained fragment I–IV or VI showed virulence to Aichi-asahi. Three out of the five transformants that contained fragment V showed avirulence toward Aichi-asahi (Table 2, Fig. 4). The virulence of the other two transformants was not determined because they could not form lesions on the leaves of Shin 2, a susceptible cultivar that lacks the *Pia* gene. These isolates might have lost their pathogenicity towards rice during transformation.

Southern analysis of Ina168 and Ina168m95-1 DNA using fragments I–VI as probes revealed that fragments I and V were single-copy DNA sequences in the genome of Ina168 and these fragments were deleted in the mutant Ina168m95-1. The four other DNA fragments hybridised with multiple bands (Fig. 5), but the hybridisation patterns of fragments II, III and VI

differed between Ina168, and the mutant by the deletion of one band. No major changes were detected in the hybridisation pattern of fragment IV, but the intensity of the band at 6 kb decreased in Ina168m95-1. The sizes of the bands that were deleted were the same as the corresponding band lengths that were calculated from the sequence data of cosmid clone 46F3. These results suggested that the DNA region that was deleted in the mutant Ina168m95-1 was larger than the insert DNA in cosmid clone 46F3, and the structure of the cosmid insert was not an artificial chimera but was conserved in the genome of strain Ina168.

In addition, we confirmed that *AVR-Pia* was located in fragment V. The fragment was introduced into strain Ina86-137, which is virulent towards Aichi-asahi, has high sporulation ability, and stable virulence (Hayashi, 2005). Seven out of 21 transformants were selected and tested for virulence towards Aichi-asahi. All the transformants acquired avirulence towards Aichi-asahi (Fig. 4). From these results, the 3.5-kb fragment V was presumed to contain *AVR-Pia*.

The 1.2-kb region Vm contains *AVR-Pia*

To localize the *AVR-Pia* gene further, 11 deleted versions of fragment V were produced by PCR using the primer sets listed in Supporting Information Tables S1 and S2. Six fragments, Va to Vf, were produced by the deletion of sequences from the 3′ end of fragment V and five fragments, Vg to Vk, were produced by deletion from the 5′ end (Fig. 6). The amplified DNA fragments were cloned into pCSN43-DEST and introduced into Ina168m95-1. A total of 72 transformants were obtained and designated as Ina168m95-1-Va to Vk. Thirty-three transformants, three transformants for each of the 11 different fragments, were selected and used for spray inoculation of Aichi-asahi. Transformants that contained fragments Va, Vb, Vc, Vd and Vg showed avirulence toward rice cultivar Aichi-asahi, whereas transformants that contained

fragments Ve, Vf, Vh, Vi, Vj and Vk showed successful infection (Fig. 6). These results indicated that fragments Va, Vb, Vc, Vd and Vg contained *AVR-Pia*, which was therefore located in the 1199-bp region that was common to all five fragments. The 1199-bp region was designated as fragment Vm. It was amplified by PCR, cloned into pCSN43-DEST, and introduced into Ina168m95-1. None of the transformants could produce susceptible lesions on Aichi-asahi (Fig. 6); therefore, the *AVR-Pia* gene was assumed to be located in this fragment.

The DNA sequence of fragment Vm contained several open reading frames (ORFs) that started with ATG (Fig. 7A). Among these, the longest ORF (255 bp), which encoded a polypeptide sequence of 85 amino acids, showed weak homology to a protein of the cytochrome c family from *Geobacter metallireducens* GS-15 (Accession no. ABB30921) with a BLAST score of 42.4 bits and E-value of 0.012. The SignalP 3.0 program (Bendtsen *et al.*, 2004) predicted an N-terminal signal peptide of 19 residues (Fig. 7B). However, there is a possibility that other gene candidates exist, because the prediction of intron–exon structure has not been performed yet.

The structure of the *AVR-Pia* locus was conserved in Japanese field isolates

DNA from 16 Japanese field isolates, eight *Pia*-avirulent (*AVR-Pia*) isolates and eight *Pia*-virulent (*avr-Pia*) isolates (Table 1), was extracted and used for Southern hybridisation with the 255-bp ORF as a probe. All eight *AVR-Pia* isolates contained a single copy of the 255-bp ORF, but the eight *avr-Pia* isolates lacked the sequence (Fig. 8). The single-copy bands that were obtained after *Hin*dIII digestion were likely to be the same as the 6.5-kb *Hin*dIII fragment derived from strain Ina168, because all these bands appeared at exactly the same position in the gel. The Vm fragments of these *AVR-Pia* strains were amplified by PCR and sequenced. The

sequences of all eight isolates were identical to the DNA sequence of Ina168 (data not shown). Therefore, it was concluded that *AVR-Pia* was located in the 1.2-kb Vm fragment, which is highly conserved among Japanese *AVR-Pia* field isolates. The same 1.2-kb DNA sequence was used for a homology search against the *M. grisea* genome database, which is available online from the BROAD Institute (http://www.broad.mit.edu/annotation/genome/magnaporthe_grisea/MultiHome.html). However, no homologous sequence could be detected in the database.

The *AVR-Pia* locus RFLP co-segregates with the phenotype in the cross described by Yasuda

AVR-Pia was also identified using a cross that utilised Chinese rice-pathogenic hermaphroditic isolates, and the chromosomal locus of the gene was identified on the linkage map using tightly-linked markers (Yasuda *et al*. 2006; Chen *et al*. 2007). In order to assess whether the DNA fragment that contains the *AVR-Pia* gene was located at the same locus in the map established independently by Yasuda *et al.* (2006), an RFLP analysis was performed on the Chinese strains using the 255-bp ORF as a probe. As shown in Fig. 9, a band of 14 kb appeared in the parental isolate that was avirulent toward Aichi-asahi, but no band was detected in the virulent parent. The presence of this band among 50 progeny that were derived from the cross between the two parental isolates co-segregated fully with avirulence towards Aichi-asahi. This result indicated strongly that the *AVR-Pia* gene cloned from isolate Ina168 was homologous to the gene in the Chinese isolates and was located at the same chromosomal locus. On the other hand, the difference in length of the hybridized bands between Ina168 and Y93-165g-1 indicated that the flanking sequence of the *AVR-Pia* locus might differ between the Japanese and Chinese isolates.

DISCUSSION

The molecular cloning of *M. oryzae* AVR genes that has been reported so far has utilised positional cloning strategies. The number of reports the difficulty associated with such strategies, namely the detailed genetic maps on which they are based have to be constructed using highly fertile genetic crosses. Rice-pathogenic *Magnaporthe* isolates are usually non-fertile (Valent *et al*., 1986, Leung *et al*., 1988). Therefore the crosses that were used for the positional cloning of AVR genes and genome analysis have utilised rare fertile rice-pathogenic isolates or laboratory strains obtained from intercrossing between non-fertile and fertile non-rice-pathogenic isolates (Valent *et al*., 1991; Valent and Chumley, 1994; Leong *et al*., 1994, Dean *et al*., 2005). In order to clone AVR genes from Japanese isolates, crosses between Japanese isolates and the fertile isolate Guy11 have been established, but the genetic map derived from these crosses is not suitable for positional cloning (Fukiya *et al*., 2001).

In the present study, a mutant-aided strategy was used in which a mutated DNA fragment that was linked closely to the gene of interest was cloned from the mutant, and therefore the poor fertility of the cross was not limiting. Crosses with low fertility are not suitable for establishing a genetic map, but can still be useful for checking whether the DNA marker co-segregates with the phenotype of interest. The successful cloning of the *AVR-Pia* locus in the present study was a rare case, because the mutated DNA fragment PM01 was found to be the only polymorphic DNA fragment identified in the RAPD analysis. One factor that simplified the identification of the PM01 fragment was that the *AVR-Pia* locus was deleted in the mutant strain Ina168m95-1; RAPD analysis is more suitable for detecting deleted DNA fragments than point mutations (Williams *et al*., 1990). It might be possible to use other methods for the detection of DNA

polymorphisms, such as amplified fragment length polymorphism (AFLP) analysis (Vos *et al*., 1995), to screen for such mutated sequences. However, a trial of AFLPs to screen for the mutated sequence in Ina168m95-1 resulted in the identification of a DNA fragment that consisted of a chimeric structure in which *Pot3* (Farman *et al.*, 1996a) had transposed onto the *MAGGY* retrotransposon (Farman *et al*., 1996b). This fragment was unsuitable for further analysis of the *AVR-Pia* locus (data not shown). Deletion events often happen at the *AVR-Pita* locus, which is located at a telomere (Orbach *et al*., 2000), and some other AVR genes have also been reported to be telomeric (Farman, 2007). Therefore, RAPD analysis might be an appropriate method for the detection of mutated DNA fragments that are associated with AVR genes.

The success of subsequent steps, after linkage between a mutated DNA fragment and the phenotype of interest has been confirmed, will depend on the distance between the DNA fragment and the gene of interest. Deletion events may vary from several hundred bps to more than 10 kb, as in the case of *AVR-Pita* (Orbach *et al*., 2000). In the present study, although the length of the deletion at the *AVR-Pia* locus was unknown, the cosmid clone 46F3 contained the DNA that is deleted in the mutant and the *AVR-Pia* gene. Genomic libraries constructed with vectors that can accommodate longer inserts, such as bacterial artificial chromosomes, may allow the marker and the gene of interest to be cloned as a single insert. Thus, this mutant-aided strategy may be applicable to the cloning of other AVR genes, with modification of the screening for the mutated DNA fragment and/or genomic library construction.

The *AVR-Pia* locus that was cloned in cosmid clone 46F3 was > 38 kb in length. The sequence revealed a mosaic structure of transposons, which were often repeated, and other DNA sequences with a lower copy number. The DNA fragments that comprised the locus may have

originated from different parts of the genome. This speculation was supported by the fact that each DNA fragment of low copy number showed homology to a different part of the genomic sequence, and, other than fragments I and V, the fragments were not present as single copies (Fig. 5). In other words, the fragments in cosmid 46F3 were duplicated forms of disparate portions of the genome. The junctions of these lower copy number DNA portions, which were derived from different origins, were transposable element insertions or transposon clusters. Chimeric structures that are related to AVR genes have also been reported for *AVR-Pik* by Luo *et al.* (2007). Therefore, such a structure might be a common feature of AVR gene loci in *M. oryzae*. Recombination events between inserted transposons or their clusters may have contributed to the construction of this chimeric structure. Such events have indeed been reported to affect the chromosomal structure of *M. oryzae* (Thon *et al.*, 2006). Recently, Khang *et al*. (2008) have reported the structure of the flanking region of the *AVR-Pita* gene family and revealed a strong relationship between the *AVR-Pita* genes and solo LTRs from RETRO6-1 and RETRO7-1. A similar structure, with two flanking solo LTRs from MGLR-3 and *Pyret* was identified in the repetitive region of the 46F3 insert (Supporting Information Fig. S1). From Southern analysis of the 46F3 insert in the mutant Ina168m95-1, the size of the deletion that occurred in Ina168m95-1 was estimated to be > 38 kb. Sone *et al*. (1997b) have reported the deletion of a large chromosomal segment that occurred at the high rate of 12.5% per sporulation in strain Ina168. However, this event did not result in a change of host cultivar specificity and thus did not contain the *AVR-Pia* locus. The relationship between the repetitive elements in the *AVR-Pia* locus and the large deletion event that occurred in Ina168m95-1 remains to be elucidated.

The *AVR-Pia* locus was mapped on the genetic maps that have been constructed independently by Yasuda *et al*. (2006) and Chen *et al*. (2007) using a fertile cross between

Chinese rice-pathogenic isolates. The putative *AVR-Pia* ORF identified in this study was located at exactly the same position as the *AVR-Pia* locus that has been identified in the fertile isolate Y93-165g-1. This locus was linked to the RFLP markers 4-178 and CH3-108H, which are located on chromosome 5 (supercontig 5.182) in the *M. oryzae* genome (Yasuda *et al*., 2006), whereas homology with tightly-linked markers in genomic supercontig 5.117 suggested that the *AVR-Pia* locus mapped by Chen *et al.* (2007) was located in the telomeric region of chromosome 7. A trial of long-range PCR using primers that were specific for the 46F3 insert, together with a telomeric primer was not successful. Thus the distance between *AVR-Pia* and the telomere remains unclear. The results of Southern analysis in this study indicated that the *AVR-Pia* genes in Japanese isolates Ina168 and Y93-165g-1 were homologous, but had different flanking regions, because the lengths of the restriction fragments that contained the *AVR-Pia* locus were distinct. Further characterization of the *AVR-Pia* locus will elucidate the structural differences between the *AVR-Pia* loci in Japanese and Chinese isolates, and also the relationship between the *AVR-Pia* locus and the telomere.

Southern analysis of virulent and avirulent Japanese isolates indicated that all the *avr-Pia* isolates lacked the Vm fragment that contained *AVR-Pia.* One interpretation of this result is that the main cause of mutation at this avirulence locus is deletion. The lack of information about the limits of the deleted region in mutant Ina168m95-1 prevented the comparative analysis of deletions among the *avr-Pia* isolates. The fact that the restriction fragment that contained *AVR-Pia* was the same in all the *AVR-Pia* isolates, and that the nucleotide sequence of the Vm fragment in these isolates was identical, indicates that the *AVR-Pia* locus has not diversified among Japanese isolates. *Pia* is one of the R genes that originated in Japanese rice cultivars and it has been maintained in most Japanese rice cultivars (Yamasaki and Kiyosawa, 1966). In the

nationwide pathotype survey that was reported by Yamada (1985), the percentage of *M. oryzae* isolates that contained *AVR-Pia* was 6% and 8% in 1976 and 1980, respectively. Due to the fact that more than 90% of the Japanese blast population lacked *AVR-Pia*, the genetic source of *AVR-Pia* should have been limited in this population. The isolates with *AVR-Pia* that were used in this study might have originated from this limited source, and thus contain identical sequences for the *AVR-Pia* locus. There is also the possibility that the *AVR-Pia* gene was introduced into an *avr-Pia* population from another non-rice pathogen such as finger millet pathogen, which has been reported to have the *AVR-Pia* gene (Yaegashi and Asaga, 1981). It will be interesting to analyse the relationships between *AVR-Pia* loci in rice pathogens or among rice and non-rice pathogens using the *AVR-Pia* sequence that was cloned in the present study.

In the present study, the *AVR-Pia* gene was localised to a 1199-bp DNA region, and the largest ORF (255 bp) is expected to be the *AVR-Pia* gene. This is supported by the fact that deletion fragments Ve and Vh, which have their 3′ and 5′ ends, respectively, in the 255-bp ORF could not complement avirulence toward *Pia* (Fig. 5). In addition, the amino acid sequence that was encoded by this ORF appeared to contain an N-terminal signal peptide. Such a sequence has been found in all other AVR gene products that have been cloned in *Magnaporthe* spp., except for *ACE1* (Böhnert *et al*., 2004). The N-terminal signal-peptide-like sequences that are present in the 255-bp ORF, *PWL2* (Sweigard *et al.*, 1995), *AVR-Pita* (Orbach *et al*., 2000), and *AVR1-CO39* (Peyyala and Farman, 2006) did not show any significant homology (data not shown). However, the presence of a signal-peptide-like sequence could be a common feature of the AVR gene products, as reported in other fungi (Kamoun, 2007). The amino acid sequence encoded by the 255-bp ORF showed weak homology to a hypothetical cytochrome c family protein from the Gram-negative bacterium, *G. metallireducens* GS-15. These proteins were not

expected to have a similar function, however, because the lengths of the amino acid sequences were very different (85 residues for the 255-bp ORF and 490 for the cytochrome c family member). The function of the product of the ORF is yet to be determined.

The DNA sequence of fragment Vm, which included the 255-bp ORF, did not show any significant similarity towards the genomic sequence of *M. grisea*. This indicated that this *AVR-Pia* gene was conserved specifically in a limited range of rice-pathogenic isolates of *M. oryzae*. In order to characterize all the AVR genes of blast fungus, genome sequence data will be important but not sufficient. Studies on different field isolates that contain a variety of AVR genes will be necessary for the completion of this goal, and the mutant-aided strategy for AVR gene cloning that is described here could make an important contribution.

EXPERIMENTAL PROCEDURES

Fungal and bacterial strains

The isolates and laboratory strains of *M. oryzae* that were used in this study are shown in Table 1. Progeny of the cross between 2107-33 and Guy11 (cross 5307 progeny; Fukiya *et al.*, 2001) and progeny of the cross between Y93-165g-1 and Y93-164a-1 (Yasuda *et al.*, 2006) were used. Fungal isolates and strains were stored at –20 °C as dried mycelium on pieces of filter paper and inoculated onto appropriate medium before use. *Escherichia coli* JM109, TOP10 (Invitrogen, Carlsbad, CA, USA), and DB3.1 (Invitrogen) were used for recombinant DNA experiments and vector construction. *E. coli* DH10B was used for the construction of the cosmid library.

Extraction and general manipulation of DNA

Fungal DNA was extracted using the method described by Sone *et al*. (1997a). Plasmid

and cosmid DNA were extracted using Quantum Prep Plasmid Miniprep or Maxiprep kits (Bio-Rad, Hercules, CA, USA). Restriction enzyme digestion of DNA was performed according to the instructions of the enzyme manufacturers. Capillary blotting for Southern hybridization was performed using Hybond-N^+ (GE Healthcare, Bucks, UK) as described in the manufacturer's instructions. Labelling and detection of DNA probes were performed with the AlkPhos Direct nucleotide labeling and detection system (GE Healthcare). DNA sequencing samples were prepared using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analyzed using an ABI PRISM 377 DNA Sequencer or ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Computer analyses of DNA sequences, including ORF analysis, were performed using the Genetyx-MAC software (Genetyx, Tokyo, Japan).

RAPD screening

The primer sets for the RAPD analysis (OPA, OPB, OPD, OPE, OPF, OPG, OPH, OPI, OPJ, OPK, OPL and OPM) were purchased from Operon Technologies (Alameda, CA, USA). RAPD amplification was performed in a reaction mixture composed of 1× PCR buffer (Applied Biosystems), 2.0 mM $MgCl_2$, 0.1 mM dNTPs, 5 ng *M. oryzae* template DNA, 2.0 μM each primer, and 0.3 U AmpliTaq DNA polymerase (Applied Biosystems) in a total volume of 12.5 μL. PCR was performed in a GeneAmp 2400 Thermal Cycler (Applied Biosystems), with 40 cycles of 94 °C for 90 s, 37 °C for 90 s, and 74 °C for 150 s. Reaction products were electrophoresed through a 1.5% agarose gel, stained with ethidium bromide, and visualised using a UV transilluminator.

Spray inoculation assay

Eight seeds from each of two rice cultivars were planted in plastic pots and grown to the fourth to fifth leaf stage. Spores were harvested by adding an appropriate amount of 0.02%

Tween-20 to plates on which the fungi had been cultured, and massaging the cultures gently with a paint brush. The spore concentration was counted using a haemocytometer, and the final spore yield was adjusted to more than 50 ml of 10^5 spores/ml. Plants were inoculated by spraying with the spore suspension. Inoculated plants were placed in a chamber at 100% humidity for 24 h at 25ºC and then transferred to a greenhouse at 20 ºC. Symptoms were scored 7 days after inoculation.

PCR sib selection of a cosmid library

The Ina168 cosmid library consisted of 4800 clones, arranged in a 50 × 96-well plate format. All of the clones were cultured overnight in 1 mL LB-ampicillin in 96-deep-well plates. The total culture medium from one plate (96 clones) was then collected in a conical tube, and the cells were harvested by centrifugation. Cosmid DNA was extracted from the culture pool and screened by PCR to detect the PM01 fragment, using the primer pair mag3 (5′-TCTGGGCGTCATTCAACCAAGCTC-3′) and mag4 (5′-GTGCTCCGAAGTTACATCACGTCC-3′). The plates from which the PM01 fragment was amplified were used for a second screening by colony hybridisation with the PM01 fragment as a probe.

Transformation

Conidia of the recipient strain were inoculated into 2YEG medium (2 g/L yeast extract, 10 g/L glucose) at 10^5 spores/100 mL and grown with rotary shaking in Erlenmeyer flasks at 27 ºC for 3 days. Mycelia were then collected on filter paper. Protoplasts were prepared from the mycelia using either 10 mg/mL lysing enzyme (Sigma–Aldrich, St. Louis, MO, USA) with SSE buffer (20% sucrose, 50 mM sodium citrate dihydrate, 50 mM EDTA, pH 8.0) or 20 mg/mL Yatalase (Ozeki, Nishinomiya, Japan) and 5 mg/mL Cellulase Onozuka (Yakult Honshya, Tokyo,

Japan) with SM buffer (0.6 M sucrose, 50 mM maleic acid monosodium salt, pH 5.5). The protoplasts were washed and stored in STC buffer (20% sucrose, 10 mM Tris–HCl, pH 7.5, 50 mM $CaCl_2$). Five to thirty micrograms of vector DNA were added to 100 μL of protoplasts (10^8 /mL) in a 50-mL conical tube, placed on ice for 20 min, and then 2 mL polyethylene glycol (PEG) 4000 (60% PEG in 10 mM Tris–HCl, pH 7.5, 50 mM $CaCl_2$) were added. This mixture was incubated on ice for 20 min and then 30 mL ice-cold STC buffer were added slowly. The protoplasts were collected by centrifugation at 3000 g for 10 min at 4 ºC. The pellet was resuspended in 1.5 mL YG1/2SC (5 g/L yeast extract, 200 g/L sucrose, 25 mM $CaCl_2$), and the suspension was added into 30 mL molten bottom agar medium (5 g/L yeast extract, 3 g/L casein hydrolysate, 10 g/L glucose, 2% agar) at 50 ºC.

The mixture was poured into two plates. Once regeneration from the protoplasts was confirmed, 15 mL of top agar medium (as for bottom agar, except 0.7% agar, and containing 800–1000 μg/ml hygromycin B [Wako Pure Chemical Industries, Osaka, Japan]) at 60 ºC was poured over the bottom agar layer. After incubation at 27 ºC for several days, the colonies that grew on the agar surface were transferred to oatmeal agar plates that contained 400–500 μg/mL hygromycin B. Single conidia were isolated from each transformant by the method described by Sone *et al.* (1997b).

Sequencing of cosmid clone 46F3

A plasmid library for cosmid clone 46F3 was prepared using a TOPO Shotgun Subcloning Kit (Invitrogen). A total of 556 plasmid clones were sequenced, and assembled using the GenomeGambler software (Xanagen, Tokyo, Japan). The gaps in the contigs were filled by performing PCR and sequencing the resultant products. The sequence of the cosmid clone was deposited into the database under accession number AB434708.

Construction of vector pCSN43-DEST

Plasmid pCSN43 (Staben *et al*., 1989) was digested with *Eco*RV (Takara Bio, Ohtsu, Japan) and dephosphorylated with Shrimp Alkaline Phosphatase (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. An Rf-A cassette (Gateway Vector Conversion Kit; Invitrogen) was ligated into the digested pCSN43 to produce pCSN43-DEST. Plasmid pCSN43-DEST contained the hygromycin phosphotransferase (*hph*) gene and Gateway cloning cassette.

Gateway subcloning into pCSN43-DEST

Based on the 46F3 insert sequence, 12 forward and 12 reverse primers were designed and synthesised. All primer sequences are described in Supporting Information Table S1 and the pairs of primers that were used for each fragment are described in Supporting Information Table S2. All PCRs were performed using KOD-plus DNA polymerase (Toyobo, Osaka, Japan). The PCR mix consisted of the following components: 1 μL KOD-plus DNA polymerase (1 U/μL), 5 μL 10× buffer for KOD-plus, 5 μL 2 mM dNTPs, 2 μL 25 mM $MgSO_4$, 1 ng template DNA, 1.5 μL each primer at 10 μM, and 33 μL distilled water. Reactions were performed in a Gene Amp® PCR system 9700 (Applied Biosystems) with the following cycling parameters: one cycle of 94 ºC for 2 min; 35 cycles at 94 ºC for 15 s, 55 ºC (61 ºC for fragment IV) for 30 s, and 68 ºC for 5 min; followed by a final extension of 68 ºC for 5 min. The PCR products for fragments I–VI were separated by electrophoresis on 1% (w/v) agarose gels in 1× TAE buffer. After the gels were stained with ethidium bromide, the desired bands were excised from the gels and purified using a GeneClean Spin Kit (Qbiogene, Irvine, CA, USA) in accordance with the manufacturer's protocol. The PCR products for fragments Va to Vk were purified using illustra MicroSpin™ S-300 HR columns (GE Healthcare). The purified PCR products were cloned individually into a

pENTR-D/TOPO vector (Invitrogen) as described in the manufacturer's protocol, to generate entry clones. The entry clones were then cloned into pCSN43-DEST by the LR reaction using LR clonase (Invitrogen) in accordance with the manufacturer's protocol.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Structure of the repetitive-sequence region in the cosmid clone 46F3. Repetitive sequence clusters are indicated with open boxes. Each component of the repetitive sequence is shown as an arrow. A different pattern was used for each type of repetitive sequence: DNA-type transposons, retrotransposons, and solo LTRs from retrotransposons are indicated by vertical stripes, slanted stripes, and checkers, respectively. *Pot3-a* and *Pot3-b* are derivatives of *Pot3* with approximately 80% identity to the original sequence (AF333034).

Table S1. List of primers used for subcloning.

Table S2. Primer pairs for the amplification of each DNA fragment.

Table 1. List of *M. oryzae* strains used in this study

Strains	*AVR-Pia*	Isolated in/description	Reference
Field isolates			
Ina168	+	Japan, 1958	Yamada, 1976
Guy11	-	French Guiana, 1978	Leung et al. 1988
Y93-165g-1	+	China, 1993	Yasuda et al. 2006
Y93-164a-1	-	China, 1993	Yasuda et al. 2006
Mu-95	+	Japan, 1993	Hayashi, 2005
Kyu89-246	-	Japan, 1989	Hayashi, 2005
Shin83-34	+	Japan, 1983	Hayashi, 2005
Ina86-137	-	Japan, 1986	Hayashi, 2005
1804-4	+	Japan, 1976	Hayashi, 2005
Ina72	+	Japan, 1956	Yamada, 1976
TH68-126	-	Japan, 1968	Hayashi, 2005
TH68-140	+	Japan, 1968	Hayashi, 2005
Ai79-142	-	Japan, 1979	Hayashi, 2005
TH69-8	+	Japan, 1969	Hayashi, 2005
Sasamori121	-	Japan, 1990	Hayashi, 2005
Ina93-3	+	Japan, 1993	Hayashi, 2005
GFOS8-1-1	-	Japan, 1993	Hayashi, 2005
P-2b	-	Japan, 1954	Hayashi, 2005
Ai74-134	-	Japan, 1974	Hayashi, 2005
Laboratory strains			
Ina168m95-1	-	Pathogenicity mutant of Ina168	This study
2107-33	+	Progeny of Ina168 x Guy11	Fukiya et al., 2000

Table 2. Virulence assay of Ina168 m95-1 transformants

Strain	Reaction of rice cultivar		*AVR-Pia* phenotype
	Shin2 (*pia*)	Aichi-asahi (*Pia*)	
Ina168	S	R	A
Ina168m95-1	S	S	V
Ina168m95-1 transformants			
Cosmids			
pMOcosX	S	S	V
cosmid 7B2	S (10/10)	S (10/10)	V
cosmid 9E12	S (10/10)	S (10/10)	V
cosmid 46F3	S (8/8)	S (5/8)	V (5/8)
		R (3/8)	A (3/8)
46F3 subclone fragments			
I	S (3/3)	S (3/3)	V
II	S (3/3)	S (3/3)	V
III	S (3/3)	S (3/3)	V
IV	S (3/3)	S (3/3)	V
V	S (3/5)	R (3/5)	A
VI	S (3/3)	S (3/3)	V

* S, susceptible; R, resistant; A, avirulent; V, virulent. Numbers in parentheses indicate the number of transformants that showed the phenotype out of the total number of transformants tested. All tests were duplicated (*n*=2).

Figure legends

Fig. 1. RAPD fragment PM01 was absent from Ina168m95-1. (A) RAPD amplification using primer OPM-01. Lane 1, Ina168; and lane 2, Ina168m95-1. An arrow indicates the PM01 fragment (1.2 kb). (B) Southern hybridization of DNA extracted from Ina168 (lane 1) and Ina168m95-1 (lane 2), using PM01 DNA as a probe. Fungal DNA was digested with *Hin*dIII, which cuts within the PM01 fragment. Note that both bands of the PM01 fragment in Ina168 were deleted in Ina168m95-1.

Fig. 2. The PM01 fragment co-segregated with pathogenicity towards rice cultivar Aichi-asahi in the progeny of cross 5307. Strain P1, 2107-33; and strain P2, Guy11. Strain numbers indicate the progeny number in cross 5307. Pathogenicity: A, avirulent; and V, virulent towards the rice cultivar Aichi-asahi, N, not determined. Southern hybridization of DNA extracted from each strain, digested with *Bam*HI, and probed with PM01 DNA is shown. The arrow indicates the band that corresponds to PM01. Sizes are given in kb.

Fig. 3. A schematic illustration of the insert in cosmid clone 46F3. Open boxes a–e indicate the single insertion of a transposon or a cluster of repetitive sequences (Supporting Information Fig. S1). The solid box indicates the location of the PM01 DNA fragment. Dotted bars indicate the regions that show significant homology with the *M. grisea* genome sequence. Numbers under the dotted bars correspond to the supercontig numbers of the homologous sequence and numbers in parentheses indicate the linkage groups of the sequences. Horizontal arrows indicate the positions of subclone fragments I–VI.

Fig. 4. Fragment V complements the *avr-Pia* phenotype of Ina168m95-1 and Ina86-137. Seedlings of rice cultivars Shin 2 (1, *pia*) and Aichi-asahi (2, *Pia*) were spray-inoculated with the conidia of the strain indicated in the upper left of each panel. Photographs were taken at 7 days after inoculation. Note that no symptoms were observed in Aichi-asahi that were inoculated with transformants of fragment V, as was also observed with the *AVR-Pia* strain Ina168.

Fig. 5. Deletion of 46F3 insert DNA in the mutant Ina168m95-1. (A) Map of the relevant restriction sites in the 46F3 insert. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; and S, *Sac*I. Open bars indicate the restriction fragments that can be detected by DNA fragments I–V (represented by filled bars) when used as probes in Southern hybridisation. The numbers on the arrows indicate the size (in kb) of each fragment. (B) Chromosomal DNA from strains Ina168 (lane 1) and Ina168m95-1 (lane 2) were digested with appropriate restriction enzymes (see below) and probed with the DNA fragments that are shown above the lane numbers. The enzymes used for the digestion were *Eco*RI (fragment I), *Bam*HI (fragment II), *Sac*I (fragment III) and *Hin*dIII (fragments IV–VI). Arrowheads indicate the hybridising bands that were deleted in Ina168m95-1.

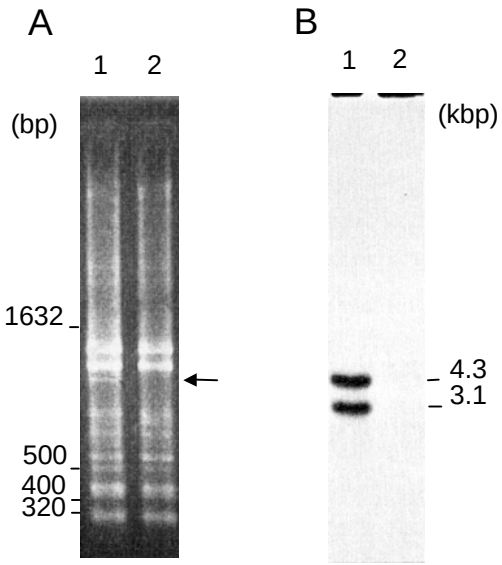
Fig. 6. *AVR-Pia* is located within fragment Vm. Schematic illustration of the deletion mutants of fragment V. Va is equivalent to fragment V. Vb to Vf were deleted from the 3′ end, and Vg to Vk were deleted from the 5′ end. Vm was derived from the inoculation results of transformants of Va to Vk, and generated by deleting from both ends of fragment V. Striped bars and open bars indicate that transformants for this fragment did or did not acquire the *AVR-Pia* phenotype,

respectively. The symptoms that appeared at 7 dpi on the leaves of cultivar Aichi-asahi after inoculation of the transformants that harboured each deletion fragment are shown to the right of the deletion mutants. Ina168 and Ina168m95-1 were the controls for the *AVR-Pia* and *avr-Pia* phenotypes, respectively. Virulence to Aichi-asahi as judged from the symptoms is shown on the righthand side. + , virulent; –, avirulent.

Fig. 7. ORF analysis of fragment Vm revealed a 255-bp ORF that encoded a polypeptide with a signal peptide and similarity to a bacterial protein. (A) ORF analysis of fragment V. The right- and left-oriented arrows indicate the ORFs identified in the 5′ to 3′ direction and the complementary direction in the sequence of fragment V, respectively. The largest 255-bp ORF is indicated. Dashed lines indicate the borders of the deleted fragments in Vm, Ve and Vb. (B) Amino acid sequence alignment of the polypeptide encoded by the 255-bp ORF with the middle part of a cytochrome c family protein from *G. metallireducens* GS-15 (Accession no. ABB30921). Identical residues are indicated with asterisks. A putative signal peptide, predicted by the SignalP algorithm, is indicated with an open box.

Fig. 8. Conservation of the *AVR-Pia* locus was investigated among Japanese field isolates. *Hin*dIII-digested DNA from each strain was probed with the 255-bp ORF DNA sequence. Lanes 1 and 14, Ina168; lane 2, Ina168m95-1; lane 3, Mu-95; lane 4, Kyu89-246; lane 5, Shin83-34; lane 6, Ina86-137; lane 7, 1804-4; lane 8, Ina72; lane 9, TH68-126; lane 10, TH68-140; lane 11, Ai79-142; lane 12, TH69-8; lane 13, Sasamori121; lane 15, Ina93-3; lane 16, GFOS8-1-1; lane 17, P-2b; lane18, Ai74-134. The pathogenicity of each isolate toward Aichi-asahi is indicated below the lane numbers. A, avirulent; and V, virulent.

Fig. 9. Analysis of co-segregation of the *AVR-Pia* phenotype and 255-bp ORF RFLP among progeny of the cross of Yasuda et al. (2006). *Hin*dIII-digested DNA from each strain was probed with the 255-bp ORF DNA sequence. P1, Ina168; N1, Ina168m95-1; N2, Y93-164a-1; and P2, Y93-165g-1. Lanes 1–50 correspond to the progeny of the cross between Y93-164a-1 and Y93-165g-1. Pathogenicity towards Aichi-asahi is shown below the lane numbers. Note that the size of the hybridising band in Y93-165g-1 was larger than that of Ina168.



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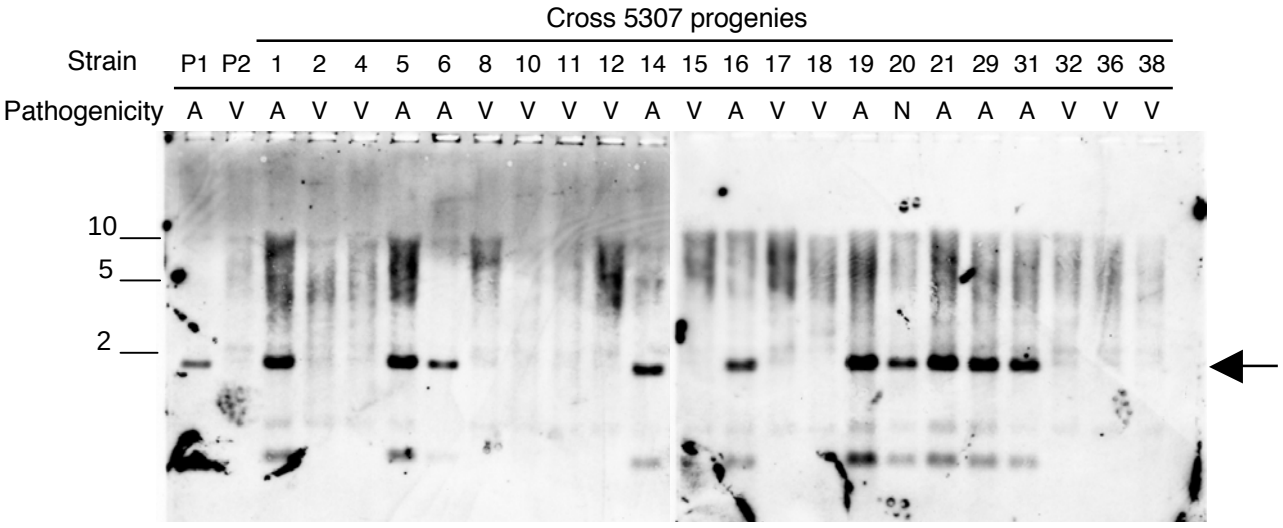

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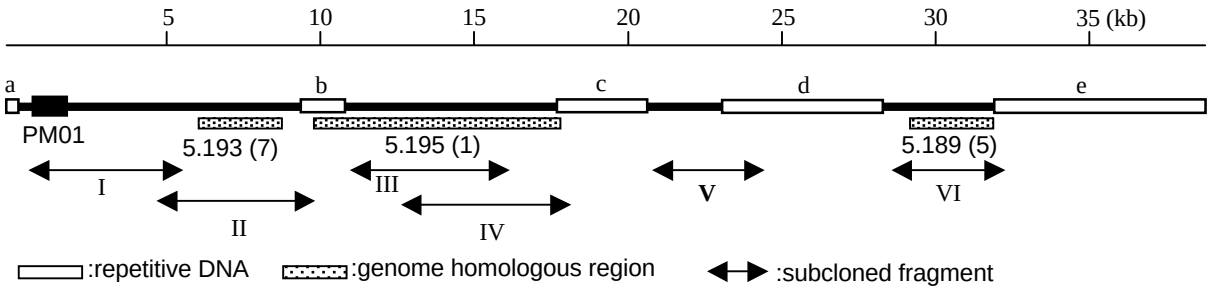

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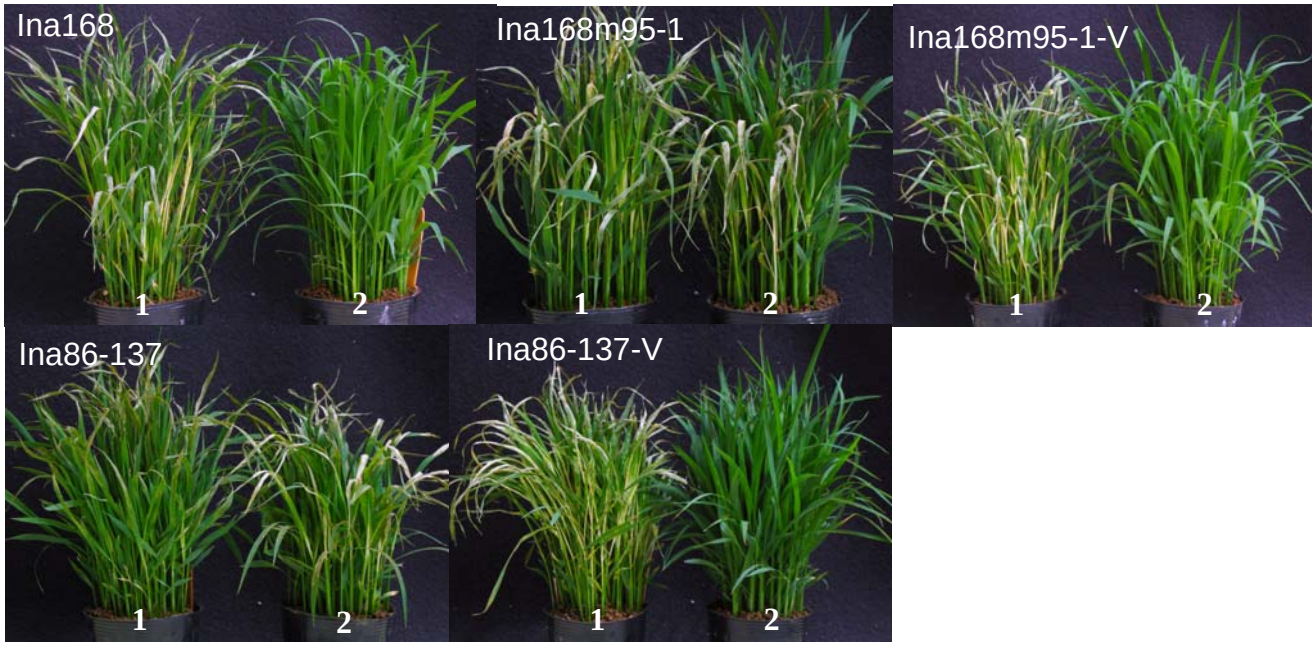

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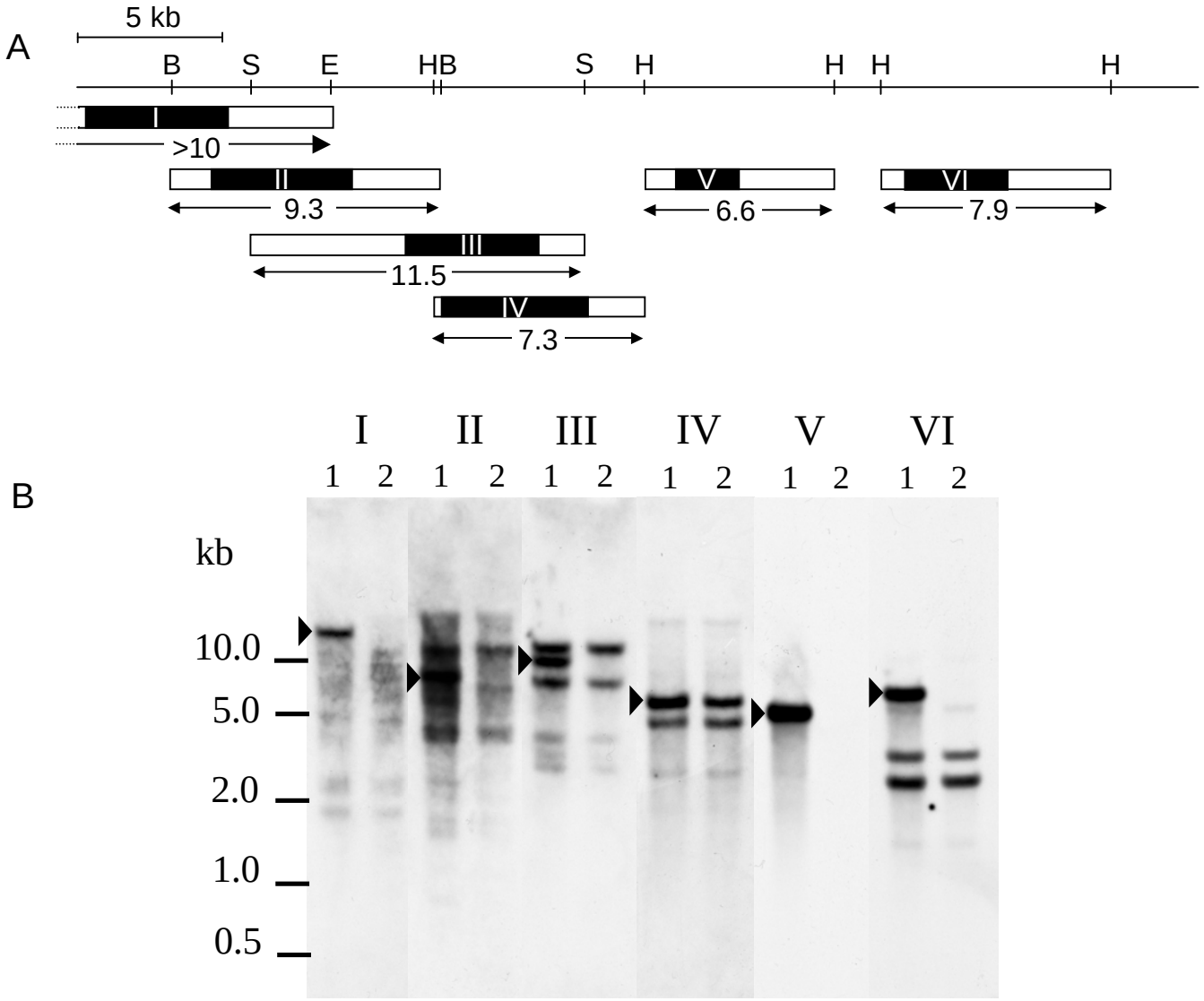

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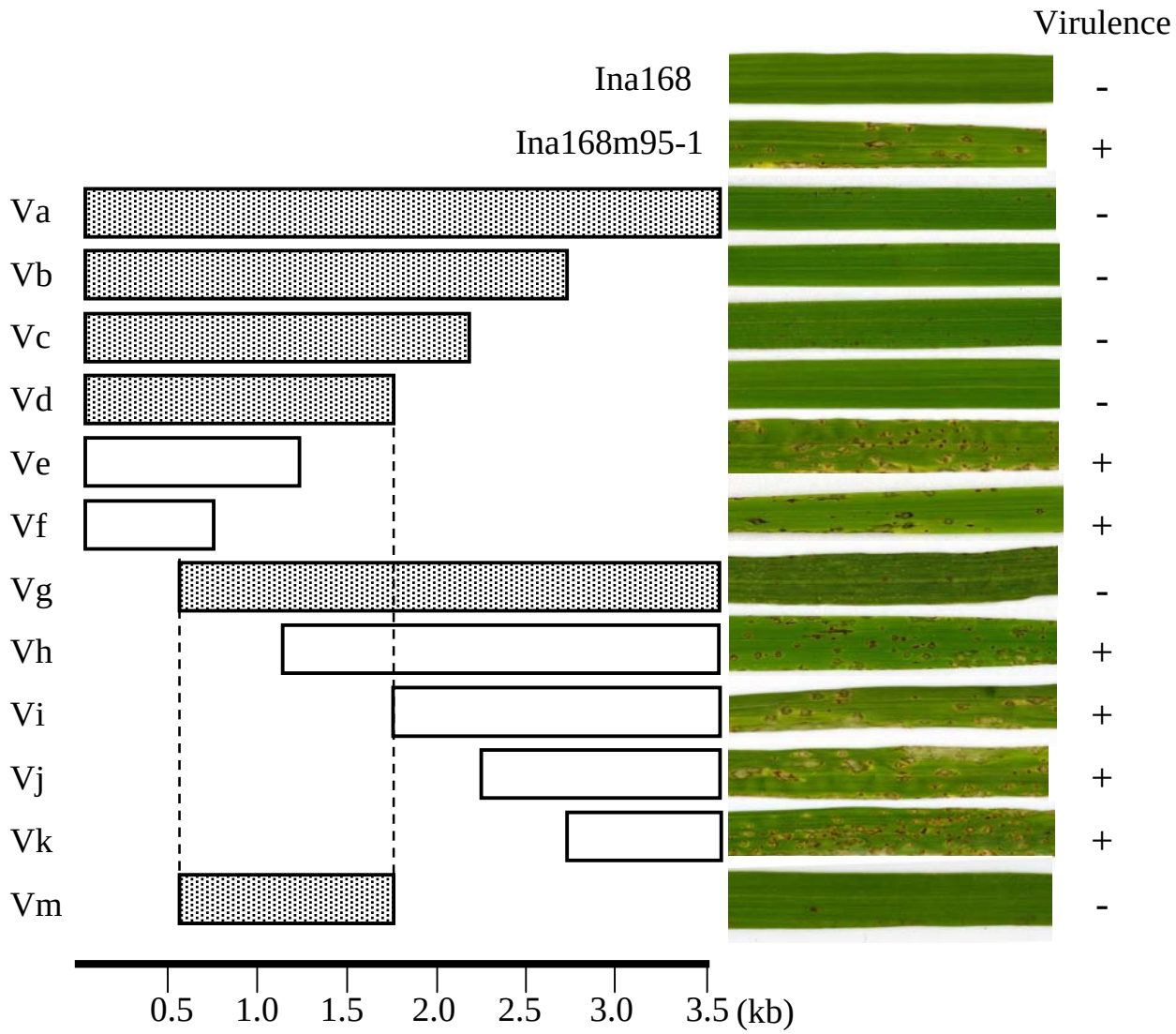

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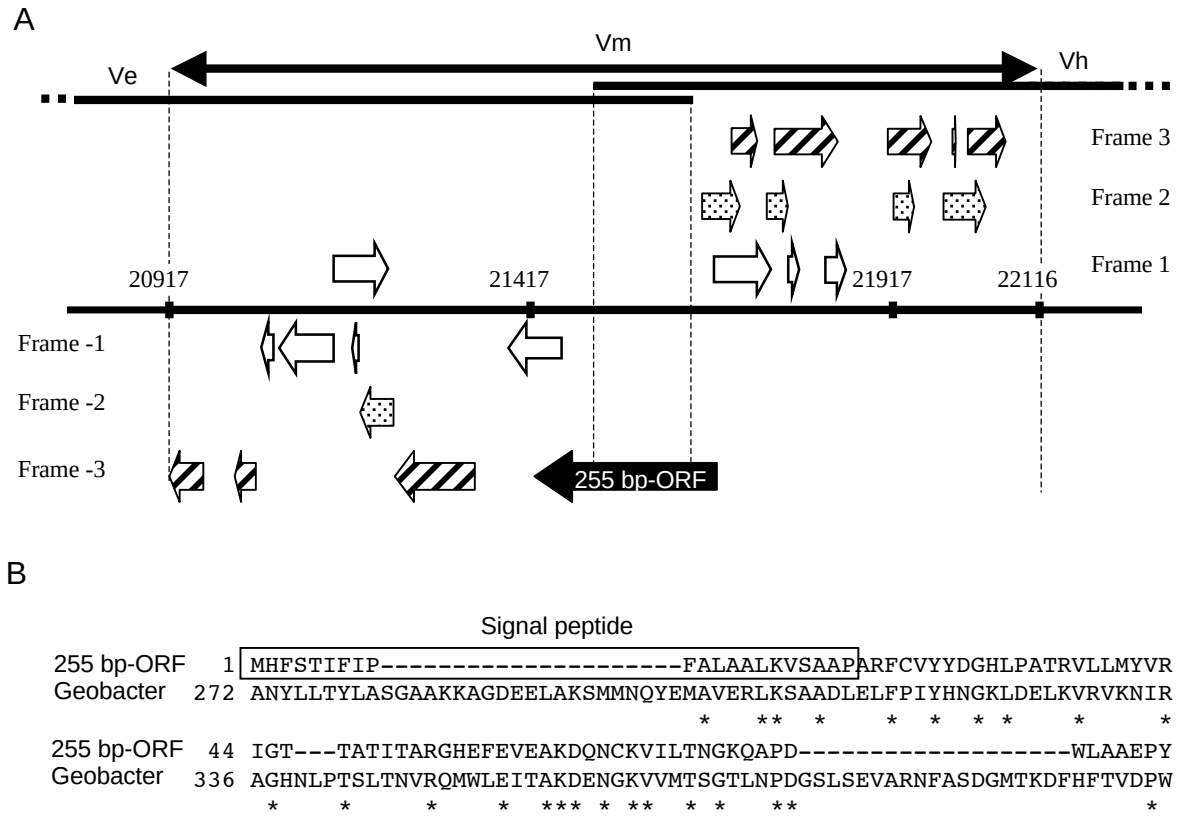

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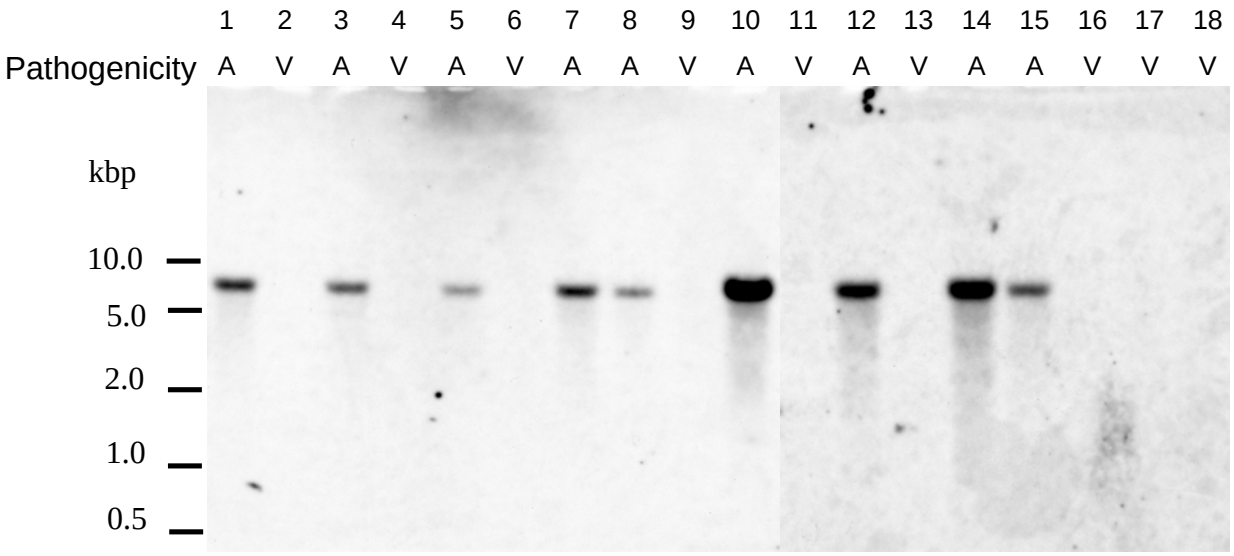

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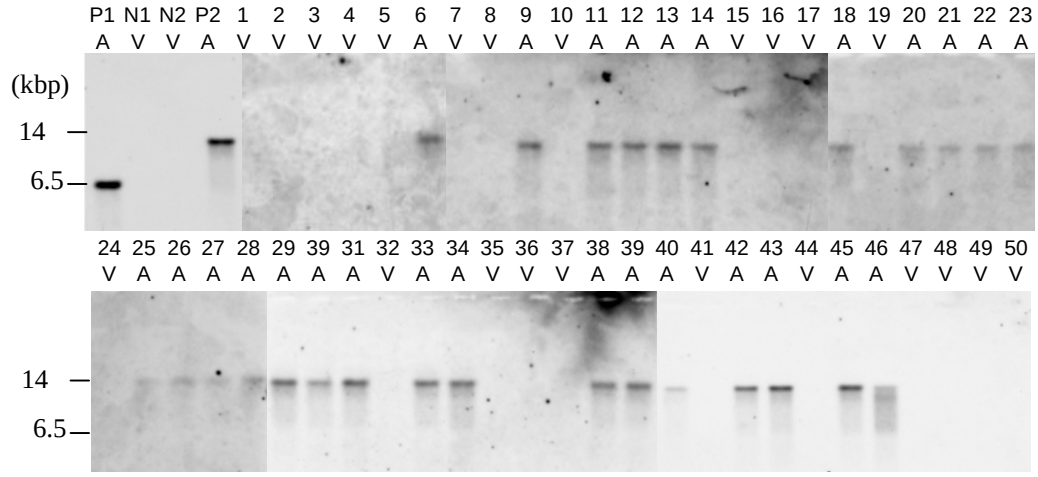

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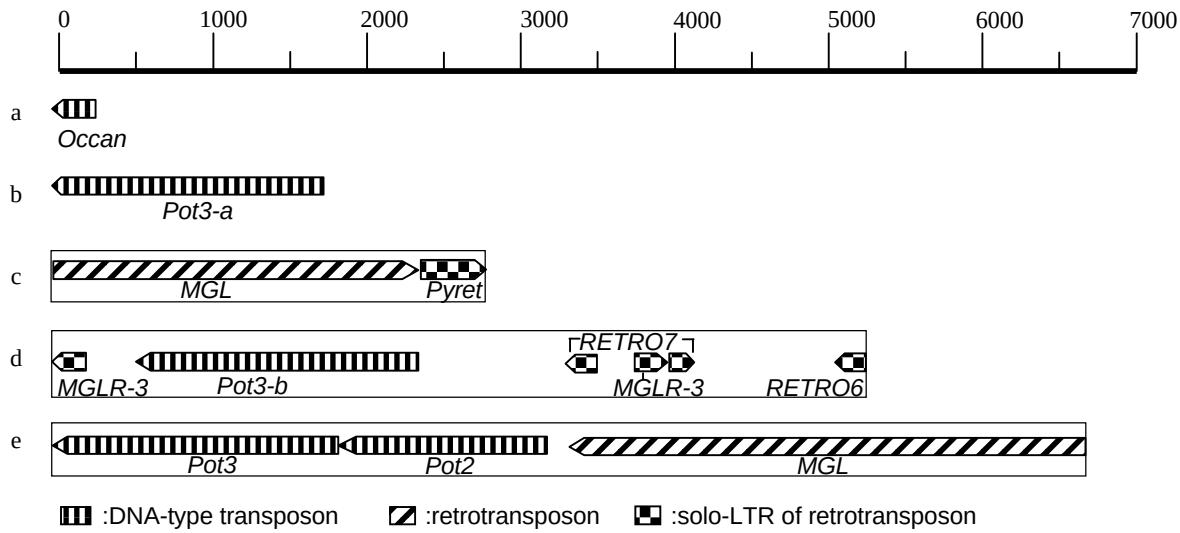

Fig. S1. Structure of the repetitive-sequence region in the cosmid clone 46F3. Repetitive sequence clusters are indicated with open boxes. Each component of the repetitive sequence is shown as an arrow. A different pattern was used for each type of repetitive sequence: DNA-type transposons, retrotransposons, and solo LTRs from retrotransposons are indicated by vertical stripes, slanted stripes, and checkers, respectively. *Pot3-a* and *Pot3-b* are derivatives of *Pot3* with approximately 80% identity to the original sequence (AF333034).

Table S1. List of primers used for subcloning

Primer name	Sequence (5’ to 3’)*
393F+cacc	caccATCTTCCATTACGCGCTCC
4668F+cacc	caccGTTTGCAGAAGCAGAGCCAA
10872F+cacc	caccGAAGCTCATCCAGCGCTAGA
12623F+cacc	caccGTACATTGCCGCAGCCAACCT
20528F+cacc	caccTAAAGCTACCCGCGCTACGT
21013F	GGGCACGTCAGAAGGCTTTA
21013F+cacc	caccGGGCACGTCAGAAGGCTTTA
21597F	TTCGAATTCGTGCCCACGGGCC
22212F	CTGGTAAGATAATGGGGCGA
22700F	ACGATAATGTTAGTGTGTTT
23175F	GGGGCGATTCTATTCGAAGA
28380F+cacc	caccCAAGTTGCTGAGCTTCACGC
5270R	GTTTTAGGGGAGCCGGAAT
9560R	AATTGCTCATCGGACCACG
15848R	TTTCTTCTCCCGGAGCAGA
17809R	CGTGGTCGTACCCTCCAGCC
21248R	GCAATCATGGCAAATTATCA
21732R	GCTCTAAAAGTAAGCGCTGC
22212R	CCAGTCGCTTGAGATTCTTTG
22680R	ATTTCGGGATGGTGTATTCT
23230R	GCGTGCCTTTCTTCTTCCTT
24040R	AGAGAACCGCAGTCTTCGGA
24040R+cacc	caccAGAGAACCGCAGTCTTCGGA
31938R	GATTCCTGGCCGTGACATCT

*Lower-case letters “cacc” indicate sequences necessary for directional cloning into pENTR/D-TOPO.

Table S2. Primer pairs for amplification of each DNA fragment

Target fragment	Primer pair (F/R)	Amplification size (bp)
I	393F+cacc/ 5270R	4896
II	4668F+cacc/ 9560R	4911
III	10872F+cacc/ 15848R	4995
IV	12623F+cacc/ 17809R	5187
V = Va	20528F+cacc/ 24040R	3532
VI	28380F+cacc/ 31938R	3578
Vb	20528F+cacc/ 23230R	2703
Vc	20528F+cacc/ 22680R	2172
Vd	20528F+cacc/ 22212R	1684
Ve	20528F+cacc/ 21732R	1205
Vf	20528F+cacc/ 21248R	721
Vg	21013F/ 24040R+cacc	3047
Vh	21597F/ 24040R+cacc	2462
Vi	22208F/ 24040R+cacc	1852
Vj	22700F/ 24040R+cacc	1360
Vk	23175F/ 24040R+cacc	885
Vm	21013F+cacc/ 22212R	1199