Identification and Characterization of Integron-Mediated Antibiotic Resistance in the Phytopathogen Xanthomonas oryzae pv. oryzae

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Abstract

Four streptomycin-resistant isolates of Xanthomonas oryzae pv. oryzae (YNA7-1, YNA10-2, YNA11-2, and YNA12-2) were examined via PCR amplification for the presence of class 1, class 2, and class 3 integrons and aadA1 and aadA2 genes, which confer resistance to streptomycin and spectinomycin. The class 1 integrase gene intI1 and the adenylyltransferase gene aadA1 were identified in all four resistant isolates but not in 25 sensitive isolates. PCR amplifications showed that 7790-bp, 7162-bp, 7790-bp, and 7240-bp resistance integrons with transposition gene modules (tni module) in 3' conserved segments existed in YNA7-1, YNA10-2, YNA11-2, and YNA12-2, respectively. Subsequent analysis of sequences indicated that the integrons of YNA7-1 and YNA11-2 carried three gene cassettes in the order |aadA3|arr3|aadA1|. The integron of YNA10-2 carried only |arr3|aadA1| gene cassettes. The integron of YNA12-2 lacked a 550-bp sequence including part of inteI1 but it still carried |aadA3|arr3|aadA1| gene cassettes. The analysis of inactive mutants and complementation tests confirmed that the aadA3 gene conferred resistance to tobramycin, kanamycin, gentamicin and netilmicin; the arr3 gene conferred resistance to rifampicin; and the aadA1 gene conferred resistance to streptomycin and spectinomycin. The resistance phenotypes of the four isolates corresponded with their resistance gene cassettes, except that YNA7-1 and YNA12-2 did not show rifampicin resistance. Sequence comparison revealed that no gene cassette array in GenBank was in the same order as in the integrons of the four resistant isolates in this study and the aadA1, which was identical in the four resistant isolates, showed 99% identity with aadA1 sequences in GenBank. The result of a stability test showed that the resistance phenotype, the aadA1 gene, and the inteI1 gene were completely stable in YNA7-1 and YNA12-2 but unstable in YNA10-2 and YNA11-2. To our knowledge, this is the first report of resistance integron in a phytopathogenic bacteria.

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Introduction

Bacterial blight of rice, caused by Xanthomonas oryzae pv. oryzae, is a serious disease in many rice-growing regions of the world, including the south of China [1–3]. Bacterial blight causes at least 10% yield loss on susceptible rice varieties when the weather is conducive [3,4].

Although the planting of resistant rice cultivars is the main approach for controlling this disease [1,3], application of streptomycin and other bactericides remains an important control method to complement the use of resistant cultivars and to reduce the emergence of resistance-breaking races [5]. Bismertiazol is the most commonly used bactericide for control of bacterial blight of rice in China [5], but bismertiazol rapidly selects for bismertiazol-resistant strains of X. oryzae pv. oryzae [6,7]. Streptomycin, an aminoglycoside antibiotic that has been widely used in treatment of bacterial diseases of humans and animals [8], is also used to control bacterial blight of rice in China [9].

Streptomycin has been used in agriculture in China for about 20 years [9]. In 2007 and 2008, 534 single-colony isolates of X. oryzae pv. oryzae were collected in the south of China to determine their susceptibility to streptomycin. The test results showed that four isolates (0.75% of the total) of X. oryzae pv. oryzae from the same county in Yunnan Province were highly resistant to streptomycin and that the resistance mechanism could not be attributed to the occurrence of strA-strB genes or to the rpsL gene mutation previously determined to cause streptomycin resistance in phytopathogenic bacteria [11–16].

Resistance integrons (mobile integrons) have been known to play important roles in acquisition and dissemination of antibiotic resistance genes [17–19]. Integrons are bacterial genetic elements that incorporate exogenous open reading frames (ORFs) by site-specific recombination and convert them to functional genes [20,21]. Integrons consist of 5' and 3' conserved segments (CS) flanking a central region containing gene cassettes. The 5' conserved region encodes three important characteristics of an integron, which are the gene for an integrase [inteI, a specific recombination site (attI) and a promoter [17,20]. There are two
major groups of integrons “chromosomal integrons” (also called superintegron) and “mobile integrons” (also called resistance integron) [17,20]. In resistance integron, the exogenous genes are usually resistance genes encoding resistance to specific antibiotics. Most resistance integrons are class 1 integrons, which have been reported in many gram-negative bacteria [17,18,22]. The ada1 gene encoding an aminoglycoside adenyltransferase inactivating streptomycin and spectinomycin [23,24], which is among the most prevalent gene in resistance integrons [23,25], has been detected in many gram-negative bacteria isolated from humans, animals, animal food products, soil [26–29], and even phylloplane bacteria [30], but no ada1 gene or resistance integron containing any other resistance gene cassette has been reported in any phytopathogenic bacteria. Here, we examined ada1 genes and int genes in streptomycin-resistant and -sensitive isolates of X. oryzae pv. oryzae by PCR amplification and found all four resistant isolates carried a class 1 integron that contained an ada1I gene cassette. In this study, the antibiotic susceptibility profiles of the four isolates were examined and analyzed. That the resistance genes in integron conferred resistance to the corresponding antibiotics was confirmed by both constructing inactive mutants and complementation tests. The stability of resistance and integrase genes was assessed.

Materials and Methods

Bacterial Strains

Bacterial strains and plasmids used in this paper are listed in Table 1. YNA7-1, YNA10-2, YNA11-2, and YNA12-2 are streptomycin-resistant and were isolated from the same county in Yunnan Province in 2007 [11]. Twenty-four isolates including YNA11-1 and YNA22-2 are streptomycin-sensitive and were randomly selected from 413 streptomycin-sensitive isolates from six provinces in the south of China in 2007, ZJ173 and PXO999 are streptomycin-sensitivity isolates maintained in our laboratory [11].

Bacterial DNA Preparation, PCR, and Nucleotide Sequencing

Template DNA was prepared using AxyPrep™ Bacterial Genomic DNA Miniprep Kit (Axygen, China). Isolates were initially characterized for streptomycin resistance-related genes including the ada1I and ada2I gene and integrase genes including the intI1, intI2, and intI3 gene by PCR. The 5’ conserved segments of the integron was characterized using primers IRI, the 3’ conserved segments was characterized using primers IRT and primer pairs for genes qacEAl1 and und1. The complete sequence of the integron was cloned with primer pairs TnIR-a and IRI, TnIR and TnIA. To amplify chromosomal integrons in X. oryzae pv. oryzae, a pair of primers ilvd-f and intgon-r was designed according to the genome sequence of X. oryzae pv. oryzae KACC10331 (GenBank accession No. AE013598). When the length of target products was shorter than 1000 bp, the 50 µl PCR mixture consisted of 2 µl of template, 5 µl of 10×PCR buffer Mg²⁺ Free (TaKaRa Taq™, TaKaRa, China), 1.25 units of Taq polymerase (TaKaRa Taq™, TaKaRa, China), 0.4 µM of each primer, 1.5 mM MgCl₂, and 200 µM of each dNTP. When the length of target products was longer than 1000 bp, the 50 µl PCR mixture consisted of 2 µl of template, 5 µl of 10×PCR buffer Mg²⁺ Free (TaKaRa LA PCR Buffer II, TaKaRa, China), 2.5 units of Taq polymerase (TaKaRa LA Taq, TaKaRa, China), 0.4 µM of each primer, 2.5 mM MgCl₂, and 400 µM of each dNTP. PCR was performed in a TaKaRa PCR Thermal Cycler Dice (TaKaRa, China). Primer pairs and conditions for amplifying were listed in Table S1 [31]. Escherichia coli JM109/pHM1 containing the adaA1 and the intH1 genes was used as a positive control when intH1 and adaA1 were amplified. Amplification products were analyzed by agarose gel electrophoresis. Amplicons were purified with the AxyPrep™ PCR cleanup kit (Axygen, USA), and the purified products were sequenced by Shanghai Sangon (Sangon, China). If necessary, amplicons were individually excised from the agarose gel and purified using the Axygene gel extraction kit (Axygen, USA) according to the manufacturer’s instructions. Following purification, the PCR products were ligated to a pMD19-T Vector (TaKaRa, China) according to the manufacturer’s recommendations and sequenced by Shanghai Sangon (Sangon, China).

Nucleotide Sequence Analysis and Accession Number

Nucleotide sequence analysis was performed by BLAST (National Center for Biotechnology Information [NCBI]) and BioEdit 7.0 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The nucleotide sequences of the resistance integrons of streptomycin-resistant isolates YNA7-1, YNA10-2, YNA11-2, and YNA12-2 were submitted to GenBank and assigned accession numbers HQ662554, HQ662555, HQ662556, and HQ662557, respectively. The nucleotide sequence of the chromosomal integrase and its flanking region from YNA11-2 was submitted to GenBank and assigned accession numbers JX998161. The accession number of the chromosomal integrase and its flanking region from ZJ173 is JX998162.

Antibiotic Susceptibility

The antibiotic susceptibility profiles of X. oryzae pv. oryzae isolates were first examined by the paper disk diffusion method, and the results were confirmed by the minimum inhibitory concentration (MIC) method. The paper disk diffusion method was carried out on Mueller–Hinton agar with 15 different antibiotic disks (Tianhe, China) according to the guidelines of the Clinical and Laboratory Standards Institute [32]. The 15 different antibiotics were tobramycin (10 µg per disk), kanamycin (30 µg per disk), rifampicin (5 µg per disk), netilmicin (30 µg per disk), gentamicin (10 µg per disk), amikacin (30 µg per disk), ampicillin (10 µg per disk), cephalothin (30 µg per disk), chloramphenicol (30 µg per disk), nitrofurantoin (300 µg per disk), neomycin (30 µg per disk), novobiocin (5 µg per disk), oxacillin (1 µg per disk), polymyxin B (300 IU per disk), and vancomycin (30 µg per disk). Escherichia coli ATCC 25922 was used as a quality control strain for the antibiotic disk diffusion method. The quality control ranges for ATCC 25922 followed the CLSI guidelines [33], while the antibiotic susceptibility results of measured isolates were determined through comparison among themselves for lack of an interpretative standard for X. oryzae pv. oryzae.

MICs were determined with a slightly modified agar dilution method [15]. Bacterial suspension in the late logarithmic growth phase was diluted to about 10⁷ CFU/ml, and 5 µl of the suspension was pipetted onto nutrient agar (NA) plates separately containing serial concentrations of streptomycin, spectinomycin, kanamycin sulfate, tobramycin, gentamicin sulfate, rifampicin, or netilmicin. All of these except netilmicin, which was obtained from Shanghai Asia Pioneer Pharmaceutical Co., Ltd. (Shanghai, China), were obtained from Bio Basic Inc. (Markham, Canada). The concentrations of antibiotic used in the MIC test included 1, 5, 10, 50, and 100 µg/ml and one additional concentration for spectinomycin (500 µg/ml), two additional concentration for streptomycin (200 and 300 µg/ml) and two additional concentrations for rifampicin (0.1 and 0.3 µg/ml). The plates were incubated at 26°C for 72 h before they were examined for
bacterial growth. The lowest concentration that completely inhibited bacterial growth was considered the MIC value.

Construction and Analysis of Resistance Gene Mutants

To reveal the contribution of the three genes (aacA3, arr3 and aadA1) in gene cassettes to antibiotic resistance, target inserted mutants were constructed through homologous single recombination [34]. To construct an aacA3 mutant, arr3 mutant and aadA1 mutant in X. oryzae pv. oryzae, a 348-bp fragment containing a partial aacA3 coding region, a 324-bp fragment containing a partial arr3 coding region, and a 355-bp fragment containing a partial aadA1 coding region were amplified through PCR from the isolate YNA11-2, then the three fragments were ligated to vector pMD18-T (TaKaRa, China), respectively. Ligation and transformation were conducted according to the manufacturer’s instructions. The fragments of PCR amplification were analyzed by agarose gel electrophoresis and the sequences of the fragments and their flanking regions in pMD18-T were identified by DNA sequencing. The three recombinant plasmids were electroporated independently into resistant isolate YNA11-2. The preparation of electrocompetent cell of YNA11-2 and electroporation performed on electroporation instrument Eppendorf Mutiporator (Eppendorf, Germany) were as described [35], except the medium was NB instead of SOC. Transformants were selected on NA plates with 100 µg/ml ampicillin. To verify transformants were correctly inserted mutants through homologous single recombination, each putative mutant was tested through PCR with two primer pairs corresponding to the inserted sequence and its flanking region (Table S1). The amplicons were analyzed by agarose gel electrophoresis and were sequenced to verify the mutants were correctly inserted. The primer pairs and conditions for amplification were listed in Table S1 and the components of PCR were as mentioned previously. The PCR amplifications were carried out in a PTC 200 thermocycler (MJ Research, Inc.). The MIC of verified mutants to antibiotics including streptomycin, spectinomycin, kanamycin sulfate, gentamicin sulfate, tobramycin, netilmicin and rifampicin were evaluated.

Function Complementation of the Gene aacA3, arr3 and aadA1 in Gene Cassettes

To further elucidate the function of the three genes (aacA3, arr3 and aadA1) in gene cassettes to antibiotic resistance, three segments

### Table 1. Bacterial strains and plasmids.

| Strain or plasmid | Relevant characteristics* | Source or reference |
|-------------------|---------------------------|---------------------|
| **Strain**        |                           |                     |
| X. oryzae pv. oryzae |                           |                     |
| YNA7-1            | Wild-type resistant isolate, containing resistance integron | Lab collection |
| YNA10-2           | Wild-type resistant isolate, containing resistance integron | Lab collection |
| YNA11-2           | Wild-type resistant isolate, containing resistance integron | Lab collection |
| YNA12-2           | Wild-type resistant isolate, containing resistance integron | Lab collection |
| YNA11-1           | Wild-type sensitive isolate | Lab collection |
| YNA22-2           | Wild-type sensitive isolate | Lab collection |
| ZJ173             | Wild-type sensitive isolate | Lab collection |
| PXO99             | Wild-type sensitive isolate | Lab collection |
| PXO1-1            | PXO99 with pUFRaacA3, Km’, Gm’/Tob’/Ntl’ | this study |
| PXO2-1            | PXO99 with pUFRarr3, Km’, Rif’ | this study |
| PXO3-1            | PXO99 with pUFRaadA1, Km’, Sm’/Sp’ | this study |
| PXO4-1            | PXO99 with pUFRintegron, Km’, Gm’/Tob’/Ntl’, Rif’, Sm’/Sp’ | this study |
| MaacA3            | aacA3 Mutant of YNA11-2, constructed through homologous recombination, Amp’, Km’/Gm’/Tob’/Ntl’* | this study |
| Marr3             | arr3 Mutant of YNA11-2, constructed through homologous recombination, Amp’, Rif’ | this study |
| MaadA1            | aadA1 Mutant of YNA11-2, constructed through homologous recombination, Amp’, Sm’/Sp’ | this study |
| **Escherichia coli** |                           |                     |
| DH5’s             | F−,Δ80d recA lacZ ΔM15 | Takara |
| **Plasmids**      |                           |                     |
| pMD18-T           |                           |                     |
| pUFR034           | Km’, IncW, Mob+, mob(p), LacZa+, PK2 replicon, cosmid | [46] |
| pUFRintegron      | A fragment containing the ORF of aacA3, arr3, aadA1 gene cloned into pUFR034 | this study |
| pUFRaacA3         | an aacA3 gene ORF cloned into pUFR034 | this study |
| pUFRarr3          | an arr3 gene ORF cloned into pUFR034 | this study |
| pUFRaadA1         | an aadA1 gene ORF cloned into pUFR034 | this study |
| aSm, streptomycin; Km, kanamycin; Gm, gentamicin; Amp, ampicillin; Rif, rifampicin; Tob, tobramycin; Ntl, netilmicin; Sp, spectinomycin; r indicates resistance to the antibiotic; s indicates susceptibility to the antibiotic. |

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of the *aacA3*, *arr3*, and *aadA1* gene and a segment of integron containing all three genes *aacA3*, *arr3*, and *aadA1*, were separately cloned to a vector and transferred to PXO99, a sensitive isolate of *X. oryzae pv. oryzae*. Three pairs of primers were designed to amplify the gene *aacA3*, *arr3*, and *aadA1* respectively from YNA11-2 and the primer pair integron and integron was designed to amplify a fragment containing all three resistance gene (*aacA3*, *arr3* and *aadA1* gene) (Table S1). The primer pairs and conditions for amplification were listed in Table S1 and the components of PCR were as mentioned previously. The PCR amplifications were carried out in a PTC 200 thermo cycler (MJ Research, Inc.). Amplification products were analyzed by agarose gel electrophoresis and sequenced by Shanghai Sangon (Sangon, China).

In order to construct recombinant plasmid, restriction enzyme site and protective bases pairs were added in the 5’terminal of primer when the primers were designed (Table S1). Plasmid pUFR034 purified with the AsyPrep™ Plasmid Miniprep Kit (Axygen, USA), and amplicons from the four primer pairs purified with the AsyPrep™ PCR cleanup kit (Axygen, USA) were double digested with SmaI and KpnI respectively. After that, amplicons were ligated to the vector pUFR034 using T4 DNA ligase (Takara, China). Four recombinant plasmids containing the gene *aacA3*, *arr3*, *aadA1* and all three were constructed respectively (designated plasmid pUFRaacA3, pUFRarr3, pUFRaadA1 and pUFR integron, Table 1).

The four recombinant plasmids pUFRaacA3, pUFRarr3, pUFRaadA1 and pUFR integron were electroporated into PXO99, respectively. The preparation of electrocompetent cells of PXO99 and electroporation were performed as described in previous section. The transformants were selected on NA plates containing 20 μg/ml kanamycin. Positive transformants were screened by PCR of target genes and further confirmed by double digestion with SmaI and KpnI of recombinant plasmids. Finally, the M/C of positive transformants to streptomycin, spectinomycin, kanamycin sulfate, gentamicin sulfate, tobramycin, netilmicin and rifampicin were examined.

### Stability Assay of the Resistance Integrons

The stability assay of the resistance integrons was performed through consecutive transfers on plates without antibiotic pressure described by Cox et al. [36]. The four resistant isolates (YNA7-1, YNA10-2, YNA11-2, and YNA12-2) harboring resistance integrons were initially transferred from stock cultures to antibiotic-free NA plates to obtain actively growing culture. One colony of each isolate was transferred to a fresh NA plate, which was incubated for 2 days at 28°C to be used as the first transfer. The bacteria on this plate were then streaked onto a fresh NA plate and incubated for 2 days at 28°C. A total of 20 successive transfers were made on NA plates without antibiotics. A full loop of bacteria needs to be used for each transfer so that the characteristic of the bacteria streaked onto the fresh plate could represent the bacteria on the source plate.

After the transferred colonies had grown on NA without antibiotic, a loop of bacteria was transferred to a fresh NA plate by plate streaking in order to obtain single colonies. Among the single colonies growing on this plate, 20 single colonies were selected for testing. The simplified M/C method with 50 μg/ml streptomycin was used to determine susceptibility to streptomycin [11]. The *intI1* and *aadA1* gene were detected by PCR as described previously except the preparation of template DNA was as described by Xu et al. [11] and 10 μl of template DNA was used instead of 2 μl of template DNA in 50 μl PCR mixture. This experiment was performed twice. In the first trial, the tests were performed on the 10th and 20th transfer. In the second trial, the tests were performed on the 5th, 10th, 15th, and 20th transfer.

### Results

#### Detection of Resistance Integrons that Contain the *aadA1* Gene Cassette Conferring Streptomycin Resistance

PCR-based examination for the presence of an integron was carried out on the four streptomycin-resistant isolates (YNA7-1, YNA10-2, YNA11-2, and YNA12-2) and three streptomycin-sensitive isolates (ZJ173, YNA11-1, and YNA22-2). The PCR amplification used oligonucleotide primers specific for three integrase genes (*intI*, which indicate the presence of an integron, and for two adenyltransferase genes (*aadK*), which confer resistance to streptomycin. No amplification products were obtained from any of these isolates when the primers specific for *intI2*, *intI3*, or *aadA2* were used (Table 2). PCR with primers specific for *intI1* and *aadA1* produced amplicons from the four resistant isolates but not from the three sensitive isolates (Table 2). To confirm the result, 22 additional sensitive isolates were screened for the presence of the *intI1* gene and the *aadA1* gene. In agreement with the negative results shown for the three sensitive isolates in Table 2, none of the 22 additional sensitive isolates showed any amplification products (data not shown), which indicated that the *aadA1* gene in the class 1 integron might contribute to the streptomycin resistance in the four resistant isolates.

#### Resistance Integron Sequences

For further characterization of these integrons, the four isolates carrying the class 1 integron were screened for the *gacΔE1* gene and the *sul1* gene, which are part of the 3’ conserved segment (CS), and for the 5′ and 3′ ends of the inverted repeat of the integron (Table 2). When amplifying the 3′ CS including *gacΔE1*, *sul1*, and 3′ inverted repeats (using primer IRT) of the class 1 integron, PCR did not yield any products with the four resistant isolates, which indicated that the 3′ CS that existed in most integrons was absent in the four resistant isolates (Table 2). Positive amplification results from resistant isolates with primers *aadA1*-R and IRI, which are specific for the 5′ end of the inverted repeat of class 1 integrons, indicated that the 5′ CS existed in the four resistant isolates. Post et al. [37] reported that some class 1 integrons contained a transposition gene module (*tni* module) of Tn402 instead of *gacΔE1* and *sul1* in the 3′ CS, the primer TniR-a (designed according to the sequence of the *tni* gene in the 3′ CS, accession number X72585) and the primer IRI in the 5′ CS were used to amplify the sequence of the resistance integron. The amplification results for the four resistant isolates using this primer pair were all positive (Figure 1, Table 2), which indicated that the 3′ CSs of the four resistant isolates were *tni* modules instead of the 3′ CSs that occur in most integrons. The complete *tni* module was amplified by the primer pair TniR and TniA (Figure 1).

The complete sequences of the resistance integrons in the four resistant isolates were analyzed through comparison and BLAST in GenBank (Table 3, Table 4, and Figure 1). The resistance integrons in YNA7-1, YNA10-2, YNA11-2, and YNA12-2 contained 7790, 7162, 7790, and 7240 bp, respectively. The sequences of the resistance integrons of YNA7-1 and YNA11-2 were identical. Compared with YNA7-1 and YNA11-2, the sequence from position 1350 to 1977 was absent in the resistance integron of YNA10-2, and the sequence from 791 to 1340 was absent in the resistance integron of YNA12-2 (Table 4 and Figure 1). The cassette array in the resistance integrons of YNA7-1

and YNA11-2 was \textit{[aacA3]arr3[aadA1]} (Table 3), in which \textit{aacA3} theoretically conferred resistance to tobramycin, kanamycin, gentamicin, and netilmicin; \textit{arr3} theoretically conferred resistance to rifampicin; and \textit{aadA1} theoretically conferred resistance to streptomycin and spectinomycin. Compared to YNA7-1, YNA10-2 lacked the \textit{aacA3} gene cassette but had the \textit{[arr3[aadA1]}] gene cassettes (Table 3 and Figure 1), and YNA12-2 lacked a 550-bp sequence, including part of the \textit{intI1} gene, but still carried the \textit{[aacA3]arr3[aadA1]} gene cassettes (Table 3 and Figure 1). The nucleotide sequences of integrons in the four resistant isolates were identical except for the absent parts.

According to BLAST searches of the complete sequences of resistance integrons, no gene cassettes of integrons in GenBank were arrayed in the same order as those in the resistance integrons

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|c|}
\hline
Isolate & \multicolumn{6}{c|}{Streptomycin susceptibility} & Gene or primer pair & \multicolumn{2}{c|}{intI-R and intI} & IRI and TnIR-a & \hline
\hline
YNA7-1 & R & + & - & - & + & - & + & + & + & \\
YNA10-2 & R & + & - & - & + & - & + & - & + & \\
YNA11-2 & R & + & - & - & + & - & + & - & + & \\
YNA12-2 & R & + & - & - & + & - & + & - & + & \\
ZI173 & S & - & - & - & - & ND & ND & ND & ND & ND & \\
YNA11-1 & S & - & - & - & - & ND & ND & ND & ND & ND & \\
YNA22-2 & S & - & - & - & - & ND & ND & ND & ND & ND & \\
\hline
\end{tabular}
\caption{PCR amplification of integron-related genes from streptomycin-sensitive and -resistant isolates of \textit{Xanthomonas oryzae pv. oryzae}.}
\end{table}

\texttt{+} and \texttt{-} indicate positive and negative PCR amplification; ND indicates not determined.

\texttt{R} indicates resistance to streptomycin and \texttt{S} indicates susceptibility to streptomycin.

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Figure 1. Structural organization of integrons in four resistant isolates of \textit{Xanthomonas oryzae pv. oryzae}. A. YNA7-1 and YNA11-2; B. YNA10-2; C. YNA12-2. The complete sequences of the resistance integrons in the four resistant isolates through PCR with primer pairs TnIR-a and IRI, TnIR and TnIA. The resistance integrons in YNA7-1, YNA10-2, YNA11-2, and YNA12-2 contained 7790, 7162, 7790, and 7240 bp, respectively. The sequence of \textit{tni} module was long and not completely shown in this figure. The sequences of the resistance integrons of YNA7-1 and YNA11-2 were identical. Compared with YNA7-1 and YNA11-2, the sequence from position 1350 to 1977 was absent in the resistance integron of YNA10-2, and the sequence from 791 to 1340 was absent in the resistance integron of YNA12-2. Location of some primers used for PCR is shown in the figure. P1 and p2 are promoter areas of integrons.

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in the four \textit{X. oryzae pv. oryzae} isolates reported here. So the resistance genes in the \textit{X. oryzae pv. oryzae} integrons were compared one by one in GenBank. BLAST analysis (Table 4) indicated that all genes in resistance integrons in \textit{X. oryzae pv. oryzae} had 100% identity with genes in GenBank except the \textit{aadA1} gene. The \textit{aadA1} gene in this study showed 99% identity with the \textit{aadA1} gene of \textit{Escherichia coli} strain 55 in the GenBank database (accession No. EF527229.1), with a substitution of G for A at position 236, which resulted in an amino acid substitution of glycine (Gly) for glutamic acid (Glu) at codon 79.

### Chromosomal Integrase Sequence

To find out the relationship between chromosomal integron and resistance integron (mobile integron) in \textit{X. oryzae pv. oryzae}, chromosomal integrase and its flanking sequence were amplified with the primer pair ivdF- and ivd2. The 3250-bp nucleotide sequences containing the chromosomal integrase gene \textit{intI1} and its flanking sequence were obtained from four resistant isolates and two sensitive isolates \textit{YNA11-1} and \textit{YNA22-2}. Sequence analysis showed that the amplified nucleotide sequences from four resistant isolates and two sensitive isolates were 100% identical, which indicated there was no direct relationship between the chromosomal integrase and the antibiotic resistance in \textit{X. oryzae pv. oryzae}. The \textit{intI1} sequence of \textit{ZJ173}, which was isolated from a different district, was 99.7% identical to the six isolates from Yunnan Province.

### Antibiotic Resistance Phenotype

The antibiotic susceptibility of the integron-carrying isolates \textit{YNA7-1}, \textit{YNA10-2}, \textit{YNA11-2}, and \textit{YNA12-2} and the non-integron-carrying isolates \textit{ZJ173}, \textit{YNA11-1}, and \textit{YNA22-2} was determined by the paper disk diffusion method and the MIC method. In the disk diffusion tests, all seven isolates were sensitive to amikacin, ampicillin, cephaptohan, chloramphenicol, neomycin, nitrofurantoin, novobiocin, polymyxin B, and vancomycin and resistant to oxacillin (resistance to oxacillin was not related to the resistance integron). Some or all of the four integron-carrying isolates were resistant to tobramycin, kanamycin, netilmicin, gentamicin, and rifampicin, while the three non-integron-carrying isolates were sensitive to these antibiotics. The MIC determination result showed that the four integron-carrying isolates were resistant to spectinomycin, while the three non-integron-carrying isolates were sensitive to spectinomycin (Table 5). In agreement with this result, the four integron-carrying isolates contained the \textit{aadA1} gene, which encodes aminoglycoside-3’-N-acetyltransferase and thereby confers resistance to both streptomycin and spectinomycin [38]. Three of the integron-carrying isolates \textit{YNA7-1}, \textit{YNA11-2}, and \textit{YNA12-2} were resistant to tobramycin, kanamycin, netilmicin, and gentamicin, while one integron-carrying isolate \textit{YNA10-2} and the three non-integron-carrying isolates were sensitive to these four antibiotics.

### Analysis and Characterization of Resistance Gene Mutants

Three resistance gene fragments including a 348-bp fragment of the \textit{aacA3} gene (designated FaccA3), a 324-bp fragment of the \textit{arr3} gene (designated Farr3) and a 355-bp fragment of the \textit{aadA1} gene (designated FaadA1) were respectively cloned into pMD18-T (Figure 2), and identified by sequencing (data not shown). The Vector pMD18-T is a suicide plasmid in \textit{X. oryzae pv. oryzae}, selection of ampicillin-resistant transformants arising from a homologous single recombination event between the plasmid pMD18-T containing the fragment of resistance gene and the genome of \textit{YNA11-2} impelled inactivation of the target resistance gene (Figure 2). The three inserted mutants were respectively identified by PCR analysis using two corresponding primer pairs. For each primer pair, one primer was located in the inserted fragment and the other primer located outside of the inserted fragment (Figure 2). The expected size of PCR amplicons was obtained in the three inserted mutants (Figure 2), while no PCR products were detected in the control parental strain \textit{YNA11-2} (data not shown). Further sequencing of PCR products confirmed that the \textit{aacA3} gene, the \textit{arr3} gene and the \textit{aadA1} gene had been mutated at the correct position (data not shown).

The MIC determination result showed that the mutant MaacA3 containing an inactivated \textit{aacA3} gene, was sensitive to kanamycin, tobramycin, netilmicin and gentamicin, while it was still resistant to streptomycin, spectinomycin and rifampicin. The mutant Marr3 carrying an inactivated copy of gene \textit{arr3}, was sensitive to rifampicin, while it was still resistant to streptomycin, spectinomycin, kanamycin, tobramycin, netilmicin and gentamicin. The mutant MaadA1 containing an inactivated \textit{aadA1} gene, was sensitive to streptomycin and spectinomycin, while it was still resistant to rifampicin, kanamycin, tobramycin,
Table 4. Predicted genes in resistance integrons in four resistant isolates of *X. oryzae* pv. *oryzae* and the alignment of these genes relative to those in GenBank.

| Gene Position | Length (bp) | YNA10-2 | YNA12-2 | Identity | Accession no. | Description of the identical gene |
|---------------|-------------|---------|---------|-----------|---------------|-----------------------------------|
| Incomplete IRi | 1–19        | 19      | Same    | Same      | 100%          | *Pseudomonas aeruginosa* isolate 96 plasmid pOZ176, Tn402-like class 1 integron, 5' CS |
| Full IRi      | 20–196      | 177     | Same    | Same      | 100%          | *Pseudomonas aeruginosa* isolate 96 plasmid pOZ176, Tn402-like class 1 integron, 5' CS |
| **intI**      | 197–1210    | 1014    | Same    | Absent    | 791–1340      | *Escherichia coli* strain IncA/C2 plasmid pRYC103T24, IntI (intI) |
| **P1 promoter area** | 1095–1123 | 29     | Same    | Absent    | 791–1340      | *Escherichia coli* strain IncA/C2 plasmid pRYC103T24, IntI (intI) |
| **P2 promoter area** | 1214–1239 | 26     | Same    | Absent    | 791–1340      | *Escherichia coli* strain IncA/C2 plasmid pRYC103T24, IntI (intI) |
| **attI**      | 1295–1352   | 58      | Absent  | 1350–1977| Same          | *Pseudomonas aeruginosa* strain PA0905 class 1 integron, partial sequence |
| **aacA3**     | 1372–1926   | 555     | Absent  | 1350–1977| Same          | *Acinetobacter baumannii* isolate K43 class 1 integron Aac6-II (aac6-II) |
| **attc for aacA3** | 1921–1980 | 60     | Absent  | 1350–1977| Same          | *Acinetobacter baumannii* isolate K43 class 1 integron Aac6-II (aac6-II) |
| **arrI**      | 2011–2463   | 453     | Same    | Same      | 100%          | *Aeromonas caviae* partial class 1 integron containing arr-3 gene. |
| **attc for arrI** | 2470–2583 | 114    | Same    | Same      | 100%          | *Aeromonas caviae* partial class 1 integron containing arr-3 gene. |
| **aadA1**     | 2587–3378   | 792     | Same    | Same      | 99%           | *Escherichia coli* strain 55 class 1 integron, aminoglycosidase adenyltransferase (aadA1) |
| **attc for aadA1** | 3380–3439 | 60     | Same    | Same      | 100%          | *Escherichia coli* strain 55 class 1 integron, aminoglycosidase adenyltransferase (aadA1) |
| **tni module** | 3536–7790   | 4255    | Same    | Same      | 100%          | *Salmonella enterica* subsp. *enterica* serovar Heidelberg plasmid pSH111_166 tni module |

*Positions were defined by the order of integron of YNA7-1 and YNA11-2.

*Maximum identity at nucleotide acid level.

*Accession no. of most similar gene in GenBank.

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netilmicin and gentamicin. These results indicated that the genes aacA3, arr3, and aadA1 were required for the resistance to kanamycin, tobramycin, netilmicin and gentamicin, rifampicin, streptomycin and spectinomycin, respectively.

Function Confirmation of the Gene aacA3, arr3 and aadA1 in Gene Cassettes

PCR amplification for the genes aacA3, arr3, and aadA1 and a segment of integron containing all three genes aacA3, arr3, aadA1, generated a 626-bp, 553-bp, 863-bp and 2389-bp DNA fragment respectively (Table S1). Sequencing results showed the fragments obtained were the target genes and the enzyme sites were successfully introduced.

Four recombinant plasmids were constructed by ligating the four fragments to the vector pUFR034 and separately introduced into PXO99 by electroporation. After being harvested from NA plates with kanamycin, positive transformants were selected through PCR. PXO1-1, PXO2-1, PXO3-1 and PXO4-1 were four positive transformants, from plasmids of which expected target gene fragments about 626-bp, 553-bp, 863-bp and 2389-bp length were amplified (Figure S1).

The MIC determination result showed that all four positive transformants were resistant to kanamycin corresponding to the vector pUFR034; PXO1-1 was resistant to gentamicin, tobramycin and netilmicin; PXO2-1 was resistant to rifampicin; PXO3-1 was resistant to streptomycin and spectinomycin; PXO4-1 was resistant to all the six antibiotics; and the resistance degree was as same as YNA11-2, except the streptomycin-resistance was slightly lower than YNA11-2 (Table 5). To sum up, PXO1-1 obtained the aaccA3 gene, showing resistance to gentamicin, tobramycin and netilmicin; PXO2-1 obtained the arr3 gene, showing resistance to rifampicin; PXO3-1 obtained the aadA1 gene, showing resistance to streptomycin and spectinomycin; and PXO4-1 obtained a segment of integron containing all three genes aacA3, arr3, aadA1, showing resistance to the six antibiotics, which confirmed the function of the genes aacA3, arr3 and aadA1 in gene cassettes.

Stability of the Resistance Integrons

The stability of the resistance integrons was assessed in the four integron-carrying isolates by growing the isolates on media without streptomycin and then assessing streptomycin resistance and determining whether the intI1 gene and the aadA1 gene were present by PCR. These results are listed in Table S2.

All transfers (the growth after each transfer on medium without streptomycin is considered a transfer) of YNA7-1 and YNA12-2 showed streptomycin resistance and retained the aadA1 gene and the intI1 gene, indicating that the integron was stable in YNA7-1 and YNA12-2 after antibiotic removal.

For YNA10-2 in the first trial, only 50% of the colonies were resistant in the 10th transfer but 100% of the colonies were resistant in the 20th transfer. The percentage of colonies containing the intI1 gene was only 11% in the 10th transfer and 0% in the 20th transfer. In the second trial, 90% of the colonies were resistant in the 5th transfer, and resistance percentage remained between 90 and 95% in the 10th, 15th and 20th transfer. The percentage of colonies containing the intI1 gene in the second trial was only 5% in the 5th transfer and was 0% by the 10th transfer. The results of the two trials indicated that, when grown in the absence of streptomycin, YNA10-2 had stable streptomycin resistance but quickly lost the intI1 gene.

For YNA11-2 in the first trial, 29% of the colonies were resistant in the 10th transfer and 0% were resistant in the 20th transfer; the intI1 gene was completely lost in the 10th and 20th transfer.

Table 5. The MIC (minimum inhibitory concentration, μg/ml) of X. oryzae pv. oryzae isolates to diverse antibiotics.

| Isolate | Antibiotic | Streptomycin | Spectinomycin | Tobramycin | Kanamycin | Netilmicin | Gentamicin | Rifampicin |
|---------|------------|--------------|---------------|------------|-----------|------------|------------|------------|
| YNA7-1  | 300        | >500         | 10            | 100        | 100       | 100        | 0.1        |
| YNA10-2 | 300        | >500         | 1             | 1          | 1         | 1          | 10         |
| YNA11-2 | 300        | >500         | 10            | 100        | 100       | 100        | 10         |
| YNA12-2 | 300        | >500         | 10            | 100        | 100       | 50         | 0.1        |
| Z1173   | 1          | 5            | 1             | 1          | 1         | 1          | 0.1        |
| YNA11-1 | 1          | 5            | 1             | 1          | 1         | 1          | 0.1        |
| YNA22-2 | 1          | 1            | 1             | 1          | 1         | 1          | 0.1        |
| PXO99   | 1          | 5            | 1             | 1          | 1         | 1          | 0.1        |
| YNA11-2**| 200        | >500         | 50            | 100        | 100       | 50         | 10         |
| PXO1-1  | ND         | ND           | 50            | >100       | 100       | 50         | ND         |
| PXO2-1  | ND         | ND           | ND            | >100       | ND        | ND         | 10         |
| PXO3-1  | 100        | >500         | ND            | >100       | ND        | ND         | ND         |
| PXO4-1  | 100        | >500         | 50            | >100       | 100       | 50         | 10         |
| MaacA3  | 100        | >500         | 1             | 1          | 1         | 1          | 5          |
| Marr3   | 50         | >500         | 50            | 50         | 100       | 50         | 0.5        |
| MaadA1  | 1          | 5            | 50            | 50         | 100       | 50         | 10         |

*YNA11-2* was an isolate of YNA11-2 which has been preserved under low-temperature for three years.

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Streptomycin Spectinomycin Tobramycin Kanamycin Netilmicin Gentamicin Rifampicin
In the second trial, 85% of the colonies were resistant in the 10th transfer but resistance percentage declined to 25% in the 20th transfer; the \textit{intI1} gene was detected in all colonies in all transfers. Overall, with respect to the streptomycin resistance of YNA11-2 the results of both trials were consistent, i.e., the resistance was gradually lost in the absence of streptomycin. The two trials differed with respect to stability of the \textit{intI1} gene in YNA11-2; the \textit{intI1} gene was completely lost by the 10th transfer in the first trial but was detected in all colonies of all transfers in the second trial.

The above results showed that the stability of streptomycin resistance differed among YNA7-1, YNA10-2, YNA11-2, and YNA12-2 and the phenotype of streptomycin resistance was completely consistent with the presence of the \textit{aadA1} gene in all tested isolates.

**Discussion**

Integrons are natural gene-capture systems that play an important role in dissemination of multistep resistance, especially in gram-negative bacteria [18,42]. The present study indicated that the class 1 integrase gene \textit{intI1} and the aminoglycoside adenylyltransferase gene \textit{aadA1} were present in all four resistant isolates but not in 25 sensitive isolates. The tests for resistance stability also demonstrated that the phenotype of streptomycin resistance differed among YNA7-1, YNA10-2, YNA11-2, and YNA12-2.
Resistance was completely consistent with the presence of the \textit{aadA1} gene in all isolates tested. The mutagenesis in three resistance genes indicated that the \textit{aacA3} gene, the \textit{arr3} gene, and the \textit{aadA1} gene were required for the resistance to kanamycin, tobramycin, netilmicin and gentamicin, rifampicin, streptomycin and spectinomycin, respectively. Complementation tests further indicated the genes \textit{aacA3}, \textit{arr3}, and \textit{aadA1} conferred resistance to corresponding antibiotic. The above results confirmed that the \textit{aadA1} gene, which is carried by the integron, confers resistance to streptomycin in field isolates of \textit{X. oryzae pv. oryzae}.

Resistance integrons have been reported in many clinical pathogens of gram-negative bacteria [18,22]. They also have been found in many genera of bacteria isolated from cattle, pigs, chickens, duck, dogs, and zoo animals [22]. Isolates carrying integrons occur not only among bacterial pathogens but also among bacteria from environmental samples, including river water, waste water, agricultural soil and manured soil, et al. [22]. Resistance integrons have also been detected in phytopathogenic bacteria (\textit{Pseudomonas spp.}) in an apple orchard [30]. Although resistance integrons are ubiquitous in bacteria from a wide variety of sources, no resistance integron had been reported from any phytopathogenic bacteria before the current report. In present study, a class 1 resistance integron containing the \textit{aadA1} gene cassette and other antibiotic resistance gene cassettes were found in \textit{X. oryzae pv. oryzae}. To our knowledge, this is the first report of a resistance integron in a phytopathogenic bacteria.

Based on the data reported here, we can make some inferences regarding the origin of the resistance integrons in the four resistant isolates of \textit{X. oryzae pv. oryzae}. Firstly, the nucleotide sequences of the four integrons were identical except for absent parts, indicating that the four integrons originated from the same source. This inference was also supported by sequences of the \textit{aadA1} genes, which were identical in the four resistant isolates but had 99% identity with the most similar \textit{aadA1} gene in the GenBank database. The difference among the nucleotide sequences of the four integrons, which was only absent parts, could be attributed to the instability of the integron. Secondly, the BLAST search revealed that no integron in GenBank had cassettes arrayed in the same order as those in the integrons in the four isolates of \textit{X. oryzae pv. oryzae}. Although similar gene cassette arrays were scarce in GenBank, it is too early to speculate about the origin of the gene cassettes in the four resistant isolates of \textit{X. oryzae pv. oryzae}. It is known that antibiotics other than streptomycin (such as rifampicin, tobramycin, gentamicin, netilmicin, and kanamycin) had not been used to control bacterial blight of rice, however streptomycin-resistant isolates contained, in addition to the \textit{aadA1}, the gene \textit{aacA3}, which confers resistance to tobramycin, gentamicin, netilmicin, and kanamycin, and the gene \textit{arr3}, which confers resistance to rifampicin. It is reasonable to speculate that the \textit{aacA3} gene cassette and the \textit{arr3} gene cassette might be simultaneously transferred together with the \textit{aadA1} gene cassette to \textit{X. oryzae pv. oryzae} when the bacteria were under the selective pressure of streptomycin. Therefore, the resistance integron of the phytopathogen \textit{X. oryzae pv. oryzae} might originate from pathogenic bacteria from humans or animals treated with antibiotics such as tobramycin, gentamicin, netilmicin, kanamycin, or rifampicin.

Gillings et al. reported chromosomal integrons existed in \textit{Xanthomonas} [43]. To understand the relationship between chromosomal integrons and mobile integrons in \textit{X. oryzae pv. oryzae}, the 3250-bp nucleotide sequences containing the chromosomal \textit{intI1} gene and its flanking region were amplified from the four resistant isolates and two sensitive isolates. The result showed that the amplified nucleotide sequences of resistant isolates and sensitive isolates were 100% identical. Though chromosomal integrons (superintegrons) are considered to be the ancestor of mobile integrons and the resistance gene cassettes according to experimental and phylogenetic data [20], the \textit{intI1} gene in resistance integron in \textit{X. oryzae pv. oryzae} was 100% identical with \textit{intI1} genes, which have been found extensively in many Gram-negative bacteria, the \textit{intI1} gene and the whole resistance integron in resistant isolates of \textit{X. oryzae pv. oryzae} should be derived from recent horizontal transfer event. Cambrey et al. recently conducted a systematic study on available integrases [44]. The results showed a significant correlation between the loss of LexA regulation and integrase inactivation and the loss of LexA regulation seemed to be normal among soil and freshwater species harboring chromosomal integrons including \textit{Xanthomonadaceae}, it meant that the chromosomal integrase in \textit{X. oryzae pv. oryzae} may be inactivated. Therefore it is believed that there was no direct relationship between the chromosomal integrase and the resistance integron in \textit{X. oryzae pv. oryzae}.

If resistance was always stable when the antibiotic pressure was absent, the risk of resistance development would be high. However, the resistance stability differed among the four integron-carrying isolates, which complicates the assessment of resistance risk. The high stability of the integron in YNA12-2 might be attributed to its incomplete integrase gene, which resulted in the non-function of integrase to integrate or excise gene cassettes; consequently, the \textit{aadA1} gene cassette was persistent and resistance was stable. The reason for the difference of stability in the other three isolates is unknown. For the sequences of integrons in the isolates YNA7-1 and YNA11-2 were 100% identical, and the unique sequence difference between YNA10-2 and YNA7-1 was the deletion of the \textit{aacA3} gene, so the difference of stability was not related to the sequence itself but probably attributed to its genetic location. The resistance integron, which is also referred as mobile integron, is usually linked to a mobile DNA element, such as a plasmid or transposon [20], but in our study the result of extraction of plasmid was uncertain (data not shown), which meant we have not determined the genetic locations of the integrons in resistant isolates of \textit{X. oryzae pv. oryzae} to date. Another interesting phenomenon was the loss of the \textit{intI1} gene, not only in YNA12-2 but also in descendants of YNA10-2 and YNA11-2. The loss of integrase gene, which is not universal in integron, is probably also related to the genetic location of integron.

The pathogenicity of the four field isolates of \textit{X. oryzae pv. oryzae}, which carried the \textit{aadA1} gene cassette on the integron conferring resistance to streptomycin, was not affected by the streptomycin resistance [11], and the stability of resistance differed among these isolates. Further studies on fitness, including growth rate and competitive ability, are needed to assess the risk of resistance.

In addition to being associated with resistance to multiple antibiotics, integrons also play a significant role in the dissemination of antibiotic resistance because of their abilities to integrate gene cassettes [45]. It is therefore possible that \textit{X. oryzae pv. oryzae} and other phytopathogen may be a new source of resistance to multiple antibiotics in the future. Conjigation assays between homologous and heterologous bacterial species should be conducted. In China the ratio of streptomycin-resistant isolates containing the \textit{aadA1} gene on the integron was very low (0.75%, 4/534) in the field [11], however, for the sampling area of Yunnan Province the percentage of resistance isolates is much higher (26.7%, 4/15). So the risk of streptomycin resistance in \textit{X. oryzae} should be paid high attention.
_X. oryzae_ and the risk of resistance integron spread through phytopathogen need more investigation and research to provide more evidence.

**Supporting Information**

**Figure S1** PCR confirmation of recombinant plasmids from transformants. M, DL2000 Marker; lane 1–4, plasmid pUFRO34, amplified by primer pairs aac6f/aac6r, arr3/arr3r, aadAf/aadAr, and integronf/integronr, respectively; lane 5, plasmid from PXO1-1, amplified by aac6f and aac6r; lane 6, plasmid from PXO2-1, amplified by arr3f and arr3r; lane 7, plasmid from PXO3-1, amplified by aadAf and aadAr; lane 8, plasmid from PXO4-1, amplified by integronf and integronr.

(TIF)

**Table S1** Primer pairs used in this study.

| Primer pair | Species/strain | Product size (bp) |
|-------------|----------------|------------------|
| aac6f/aac6r | _X. oryzae_ pv. _oryzae_ | 1158–1164 |
| arr3/arr3r  | _X. oryzae_ pv. _oryzae_ | 1158–1164 |
| aadAf/aadAr | _X. oryzae_ pv. _oryzae_ | 1158–1164 |
| integronf/integronr | _X. oryzae_ pv. _oryzae_ | 1158–1164 |

**Table S2** Stability of streptomycin-resistance phenotype and the _aadA1_ and _intI1_ gene in resistant isolates of _X. oryzae_ pv. _oryzae_.

| Strain | Day 1 | Day 7 | Day 14 | Day 21 |
|--------|-------|-------|--------|--------|
| PXO1   | 9.8   | 9.7   | 9.6    | 9.5    |
| PXO2   | 9.9   | 9.8   | 9.7    | 9.6    |

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**Author Contributions**

Conceived and designed the experiments: MGZ YX. Performed the experiments: YX QQL. Analyzed the data: YX QQL. Contributed reagents/materials/analysis tools: YX QQL. Wrote the paper: YX QQL MGZ.

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