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A.H. Sha · X.H. Lin · J.B. Huang · D.P. Zhang

Analysis of DNA methylation related to rice adult plant resistance to bacterial blight based on methylation-sensitive AFLP (MSAP) analysis

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Abstract DNA methylation is known to play an important role in the regulation of gene expression in eukaryotes. The rice cultivar Wase Aikoku 3 becomes resistant to the blight pathogen *Xanthomonas oryzae* pv. *oryzae* at the adult stage. Using methylation-sensitive amplified polymorphism (MSAP) analysis, we compared the patterns of cytosine methylation in seedlings and adult plants of the rice cultivar Wase Aikoku 3 that had been inoculated with the pathogen *Xanthomonas oryzae* pv. *oryzae*, subjected to mock inoculation or left untreated. In all, 2000 DNA fragments, each representing a recognition site cleaved by either or both of two isoschizomers, were amplified using 60 pairs of selective primers. A total of 380 sites were found to be methylated. Of these, 45 showed differential cytosine methylation among the seedlings and adult plants subjected to different treatments, and overall levels of methylation were higher in adult plants than in seedlings. All polymorphic fragments were sequenced, and six showed homology to genes that code for products of known function. Northern analysis of three fragments indicated that their expression varied with methylation pattern, with hypermethylation being correlated with repression of transcription, as expected. The results suggest that significant differences in cytosine methylation exist between seedlings and adult plants, and that hypermethylation or hypomethylation of specific genes may be involved in the development of adult plant resistance (APR) in rice plants.

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A.H. Sha · X.H. Lin · J.B. Huang · D.P. Zhang (✉)
National Key Laboratory of Crop Genetic Improvement,
National Center of Crop Molecular Breeding,
Huazhong Agricultural University, Wuhan, 430070, China
E-mail: dpzhang@mail.hzau.edu.cn
Tel.: +86-27-87282031
Fax: +86-27-87396734

Present address: A.H. Sha
Institute of Oil Crop Research,
Chinese Academy of Agricultural Sciences,
Wuhan, 430062, China

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Introduction

DNA methylation has been implicated in gene regulation, transgene silencing and genomic imprinting, as well as in the control of parasitic DNA elements in plants (Wassenegger 2000). Methylation generally represses transcription (Finnegan et al. 2000). DNA methylation regulates gene expression at two levels, by transcriptional gene silencing (TGS) and by posttranscriptional gene silencing (PTGS). The former involves the inhibition of transcription and is associated with the hypermethylation of promoter sequences, while the latter involves the posttranscriptional degradation of RNA species, does not affect transcription rate and is associated with the hypermethylation of transcribed or coding sequences (Paszkowski and Whitham 2001). In plants, methylcytosine usually occurs in both CpG and CpNpG sequences, and the methylation state can be maintained through cycles of DNA replication and is likely to play an integral role in regulating gene expression (Wassenegger 2000). Two methods are routinely used for the detection of DNA methylation in the tissues of eukaryotic organisms. These depend on the application of bisulfites or methylation-sensitive restriction enzymes. Bisulfites convert unmethylated cytosine into thymine, thus allowing the detection of cytosine methylation. Some restriction enzymes (Isoschizomers) share the same recognition sites but show differential sensitivity to DNA methylation. Thus, polymorphic DNA fragments can be generated after digestion of methylated genomic DNA with isoschizomers (Xu et al. 2000). Methylation-sensitive amplified polymorphism (MSAP) analysis is based on the use of isoschizomers for detection of DNA methylation. It is an adaptation of the amplified fragment length polymorphism (AFLP) technique (Reyna-Lopez et al. 1997), in which the isoschizomers *Hpa*II and

*Msp*I are employed as 'frequent-cutter' enzymes for AFLP, instead of the usual *Mse*I. *Hpa*II and *Msp*I recognize the same tetranucleotide sequence (5′-CCGG-3′), but display differential sensitivity to DNA methylation. *Hpa*II is inactive when either of the two cytosines is fully methylated, but cleaves hemi-methylated 5′-CCGG-3′ at a lower rate than the unmethylated sequence. *Msp*I cleaves 5′-C^{5m}CGG-3′, but not 5′-^{5m}CCGG-3′.

Adult plant resistance (APR) is a type of disease resistance that becomes effective only after the plant has developed to a specific stage. To date, little is known about the mechanism of APR. The rice (*Oryza sativa*) cultivar Wase Aikoku 3 shows APR to bacterial blight (caused by *X. oryzae* pv. *oryzae*) and the APR is controlled by the resistance gene (R gene) *Xa3* (Zhang et al. 1984; Zhao et al. 1987). To investigate whether changes in DNA methylation might be involved in conferring APR, MSAP is used to assay the methylation status of genomic DNA in seedlings and adult plants of Wase Aikoku 3 that had been inoculated with *X. oryzae* pv. *oryzae*, subjected to mock inoculation or left untreated.

Materials and methods

Bacterial and plant maintenance and inoculation

X. oryzae pv. *oryzae* strain T7133 was subcultured at 28°C for 72 h on slants of Wakimoto's agar. Inocula were adjusted to a concentration of 10^9 cells/ml with distilled water.

Seeds of cv. Wase Aikoku 3 were sown in seed boxes in the greenhouse after germination. Seedlings were transplanted to a paddy field 14 days after seeding. Fully expanded leaves were inoculated with T7133 at the seedling (seventh leaf) stage and at the adult (13th leaf) stage, by using scissors dipped in bacterial suspensions to clip leaves 1–2 cm below the tip of the leaf blade. Mock-infected plants were treated in a similar fashion, except that water was used to wet the scissors. After 2–3 weeks, the distance from the cut surface at the tip to the distal-most position that exhibited a gray, chlorotic or water-soaked lesion was measured and defined as the lesion length. Plants were defined as resistant if the lesion length on leaves was less than 6 cm.

Methylation-sensitive amplification polymorphism (MSAP) analysis

Total genomic DNA was extracted from rice leaves of seedlings and adult plants that had been mock infected, left untreated, or inoculated with T7133 according to the protocol developed by Chen and Ronald (1999). The MSAP was adapted from Xu et al. (2000). Aliquots (500 ng) of DNA were digested for 2.5 h at 37°C with 5 U each of *Eco*RI and *Hpa*II (MBI) in 25 µl of 1Y$^+$/TANGO (MBI) buffer. In the second reaction, the same amount of rice genomic DNA was digested with *Eco*RI and *Msp*I under the same reaction conditions. The DNA fragments from the two reactions were added separately to an equal volume of the adapter/ligation solution, and the ligation reaction was allowed to proceed overnight at 37°C. The ligation mixture was then diluted 1:10 dilution with TE, and used as the template for the preselective amplification with *Eco*RI + A and *Hpa*II/*Msp*I + T primers. The reaction was performed for 25 cycles of 30 s denaturation at 94°C, 1 min annealing at 56°C, and 1 min extension at 72°C. The product was diluted 20-fold (v:v) with TE buffer, and used as the template for the selective amplification reaction. In this step, *Eco*RI and *Hpa*II/*Msp*I primers with two additional selective nucleotides were used. The selective PCR was performed in a final volume of 10 µl following the protocol of Vos et al. (1995). The products of selective amplification were resolved by electrophoresis on 6% sequencing gels and stained with silver (Chalhoub et al. 1997).

Isolation and characterization of amplified fragments

Amplified fragments were excised from the gel with a razor blade. The gel slices were then hydrated in 100 µl of water and incubated at 95°C for 15 min. The eluted DNA was amplified with the same primers under the conditions used for selective amplification. Sequence information was obtained by cloning the fragments in the pGEM-T easy vector (Promega, Madison, WI, USA) and sequencing individual clones. The sequences obtained were compared with nucleotide sequences in the publicly available databases using BLAST.

RNA isolation and Northern analysis

Leaves of seedlings and adult plants that had been inoculated with *X. oryzae* pv. *oryzae* strain T7133, mock-infected or left untreated were harvested at 48 h. Total RNA was isolated with the Tripure Isolation Reagent (Roche) according to the manufacturer's instructions. Samples (20 µg) of total RNA were fractionated on a denaturing 1% agarose gel containing formaldehyde, transferred to Hybond-N$^+$ membrane, and hybridized with a DIG-labelled probe according to the manufacturer's instructions (Roche Applied Science, USA). Total RNA was quantified by measuring the A_{260} value as an indication of the relative amounts of RNA loaded in each lane. Ribosomal RNA in the gels was visualized by staining with ethidium bromide and photographed on a UV trans-illuminator to serve as a further loading control.

Results

Resistance of Wase Aikoku 3 to *X. oryzae* pv. *oryzae* strain T7133

Lesion lengths were measured on leaves 2 and 3 weeks after inoculation at the seedling and adult stages,

respectively. The average lesion length on leaves of seedlings inoculated with T7133 was more than 8 cm, while it was only 1–2 cm on those of adult plants. No symptoms were observed in either seedlings or adult plants leaves inoculated with water. These results indicate that Wase Aikoku 3 is resistant to T7133 at the adult stage but susceptible to infection at the seedling stage.

Cytosine methylation profiles in inoculated and uninfected seedlings and adult plants

Since *Hpa*II is inactive when either of the two cytosines is fully methylated whereas *Msp*I is sensitive only to methylation at the external cytosine, methylation of the internal cytosine would lead to the appearance of a fragment in the amplification product generated from the *Eco*RI/*Msp*I digest but not in that obtained from the *Eco*RI/*Hpa*II digest. Similarly, hemimethylation of either of the two cytosines would lead to the appearance of a fragment in the amplification product from the *Eco*RI/*Hpa*II digest but not the *Eco*RI/*Msp*I digest. We used 60 pairs of primers to detect cytosine methylation at 5′-CCGG-3′ in the genomic DNAs of seedlings and adult plants that had been subjected to the different treatments described above. Each of the fragments represented a recognition site cleaved by one or both of the isoschizomers. Of the 2000 fragments detected, 380 were differentially amplified from the two digests in the case of at least one of the treatments (Table 1). Of these, 301 were due to hemimethylation of the external cytosine, which permits cleavage by *Hpa*II but not by *Msp*I, whereas the remaining 79 were due to full methylation of the internal cytosine, which allows cleavage by *Msp*I but not by *Hpa*II.

Differences in mehtylation patterns among inoculated and uninfected seedlings and adult plants

The patterns of bands amplified from seedlings and adult plants that had been inoculated or not with *X. oryzae* pv. *oryzae* were compared, in order to identify cases of differential amplification (Fig. 1, Table 2). Four major classes of banding patterns were identified among the differentially amplified fragments (Table 2). In the first class (class A), the same methylation sites were detected in all adult plants but not in seedlings. These represent sites that are hypermethylated specifically in adult plants, and comprise 22 hemi-methylated and 11 fully methylated sites. In the second class (class B), the methylated sites were detected in all adult plants and seedlings, with the exception of the seedlings that had been inoculated with the pathogen. These comprised six hemi-methylated sites and one fully methylated site, and represent sites that undergo demethylation in seedlings in association with exposure to the pathogen. C-type sites were methylated in all seedlings but not in any of the adult plants. These comprised two fullly methylated sites, and represent sites that are hypermethylated specifically in seedlings. The methylated sites in class D were detected in all seedlings and adult plants, with the exception of the adult plants that had been inoculated with *X. oryzae* pv. *oryzae*. This set comprised three hemi-methylated sites, at which demethylation occurs in adult plants specifically in association with exposure to the pathogen.

Analysis of polymorphic fragment sequences

To obtain more information about the sequences that are targeted by MSAP analysis, we isolated and sequenced all fragments that were differentially amplified

Table 1 Summary of polymorphism due to variation in methylation state detected in seedlings and adult plants inoculated with *X. oryzae* pv. *oryzae*, mock-inoculated or left untreated

Primer[a]	HM + TTC		HM + TAC		HM + TGC		HM + TAG		HM + TTG	
	H	M	H	M	H	M	H	M	H	M
E + AAC	1	4	1	7	0	7	2	0	2	13
E + AAA	0	5	3	12	2	4	3	4	3	4
E + AAT	3	4	4	10	0	5	1	1	2	6
E + AAG	3	3	1	9	3	6	0	2	1	6
E + ACT	4	7	0	8	1	2	3	3	1	7
E + ACC	2	5	0	11	0	4	2	2	3	6
E + ACA	0	5	0	11	0	8	1	2	0	6
E + ACG	0	2	2	3	2	1	2	4	1	6
E + AGT	0	2	1	9	1	2	1	5	0	6
E + AGC	0	6	1	9	1	3	1	4	2	2
E + AGG	4	4	1	1	0	8	0	4	3	13
E + AGA	3	6	1	1	0	3	0	6	0	2
Total[b]	20	53	15	91	10	53	16	37	18	77

[a]The five HM + 3 primers indicated across the top were used in combination with the 12 E + 3 primers for selective amplification. The core sequence of the *Hpa*II-*Msp*I and *Eco*RI primers was exactly the same as that used by Xu et al. (2000). *H* and *M* refer to fragments amplified from the products digested with *Eco*RI + *Hpa*II and *Eco*RI + *Msp*I, respectively

[b]In all, 380 differentially amplified fragments were identified

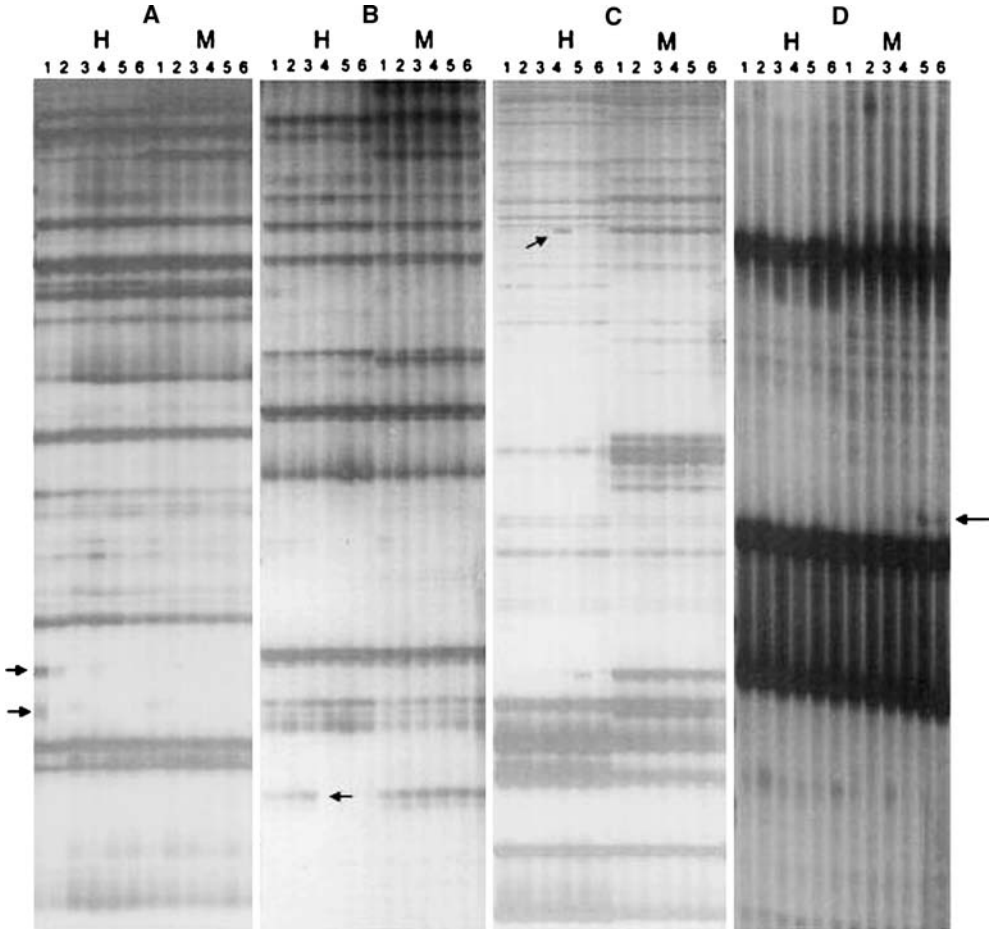

Fig. 1 **A**–**D** Methylation-sensitive AFLP analysis of seedlings and adult plants inoculated with *X. oryzae* pv. *oryzae* strain T7133, mock-infected or left untreated. DNA fingerprints were generated with the primer combinations *Eco*RI + AGA/*Hpa*II-*Msp*I + TTC (**A**), *Eco*RI + ACT/*Hpa*II-*Msp*I + TAC (**B**), *Eco*RI + ACT/*Hpa*II-*Msp*I + TAG (**C**) and *Eco*RI + ACG/*Hpa*II-*Msp*I + TAC (**D**). H and M refer to digestion with *Eco*RI + *Hpa*II and *Eco*RI + *Msp*I, respectively. *Lanes 1 and 4* plants inoculated with *X. oryzae* pv. *oryzae* strain T7133; *lanes 2 and 5* plants inoculated with water (mock-infected); *lanes 3 and 6* untreated plants. *Lanes 1–3* show patterns from seedlings and *lanes 4–6* are from adult plants. The *arrows* indicated polymorphic bands

between seedlings and adult plants in association with the different treatments. Homology was detected in GenBank for 25 fragments (Table 3). The remaining fragments were either also homologous to one or other of these 25 or could not be cloned. Of the 25 fragments, six show homology to genes whose functions are known, seventeen have homology to a genomic DNA clone or predicted mRNA, and no significant similarity was found for the remaining two (Table 3). The genes with known functions encode a Gag-Pol polyprotein (A1), the hypothetical protein PA2929 (A2), a homolog of CD8 antigen beta polypeptide (A3), a β-1,4 endoglucanase (A4), an RNA helicase of the Ski2 subfamily (B1), and a receptor-like protein kinase (C1), respectively (Table 3).

Table 2 Patterns of cytosine methylation in seedlings and adult plants inoculated with *X. oryzae* pv. *oryzae*, mock-inoculated or left untreated

Pattern[a]	H	M	H	M	H	M	H	M
A1	22							
A2		11						
B1			6					
B2				1				
C						2		
D							3	

[a]Class A patterns reveal hypomethylation in all seedlings, irrespective of how these were treated. *Class B* patterns reveal hypomethylation only in the seedlings that had been inoculated with *X. oryzae* pv. *oryzae* strain T7133. *Type C* reveals hypomethylation in all adult plants tested. The *D-type* pattern detects hypomethylation only in adult plants inoculated with the pathogen. *H* and *M* refer to fragments amplified from the product digested with *Eco*RI + *Hpa*II and *Eco*RI + *Msp*I, respectively

Expression of polymorphic fragments

To investigate whether modification of the methylation status alters the expression of functional genes, the expression of three polymorphic fragments (A1, B1 and C1; see previous section and Table 3) was analyzed on Northern blots (Fig. 2). All three fragments showed differences in expression that were correlated with their methylation state. Fragment A1(hypomethylated in all seedlings) was expressed more strongly in seedlings than in adult plants, regardless of whether or not they were infected with the pathogen. Fragment B1 (hypomethylated in infected seedlings) was expressed much more strongly in seedlings inoculated with *X. oryzae* pv. *oryzae* strain T7133 than in those that were not infected, and the fragment C1 (hypomethylated in all adult plants) was expressed in adult plants more strongly than in seedlings, regardless of how they were treated (Fig. 2).

Table 3 Sequences of AFLP fragments homologous to those in the databases

Fragment[a]	Restriction pattern[b]	Length (bp)	GenBank Accession No.	Sequence homology
A1	H	290	gi 37536916	Putative Gag-Pol polyprotein (*Oryza sativa*)
A2	M	261	gi 15598125	Hypothetical protein PA2929 (*Pseudomonas aeruginosa*)
A3	M	100	gi 29789806	CD8 antigen beta polypeptide (*Homo sapiens*)
A4	M	213	gi 505195	Beta-1,4 endoglucanase (*Humicola*)
A5	H	308	gi 50900733	Predicted mRNA (*O. sativa*)
A6	H	228	gi 32487318	*O. sativa* chromosome 4, BAC OSJNBa0061C08
A7	H	233	gi 40253725	*O. sativa* clone OSJNBb0003H03
A8	H	133	gi 40253451	*O. sativa* chromosome 8, PAC clone P0709D11
A9	H	228	gi 31431454	*O. sativa* chromosome 10 section 36 of 77 of the complete sequence
A10	M	130	gi 38569179	*O. sativa* chromosome 4, BAC clone OSJNBa0086B14
A11	H	233	gi 40253725	*O. sativa* chromosome 8, BAC clone OSJNBb0003H03
A12	H	159	gi 45725466	*Danio rerio* clone DKEY-151G22
A13	H	201	gi 21327632	*H. sapiens* BAC clone RP11-729M20
A14	H	201	gi 21327632	*H. sapiens* BAC clone RP11-729M20
A15	M	104	gi 41615378	*Rumex acetosa* Y chromosome specific sequence DOP-PCR/AFLP
A16	H	149		No significant similarity
A17	H	101		No significant similarity
B1	H	308	gi 19173634	Putative RNA helicase of the Ski2 subfamily (*Encephalitozoon cuniculi*)
B2	M	138	gi 31414502	*O. sativa* chromosome 12, BAC clone OSJNBa0018E22
B3	H	198	gi 23396275	*H. sapiens* chromosome 3 clone RP11-27C2
C1	M	127	gi 37531774	Putative receptor-like protein kinase (*O. sativa*)
C2	M	172	gi 38347817	*O. sativa* chromosome 8, PAC clone P0470B03
D1	H	332	gi 34908040	*O. sativa* PAC clone P0506B129
D2	H	328	gi 33667142	*O. sativa* chromosome 3, BAC OSJNBa0027H16
D3	H	189	gi 52208053	*Burkholderia pseudomallei* strain K96243 chromosome 1

[a]Fragments amplified from the products digested with *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I were cloned and sequenced. The sequence information was used to query the GenBank database using BLASTn. *Fragments A1–17* were amplified from all seedlings, irrespective of whether or not they had been inoculated with *X. oryzae* pv. *oryzae* strain T7133. *Fragments B1–3* were amplified only from seedlings that had been inoculated with the pathogen. *Fragments C1 and C2* were amplified from all adult plants tested, *D1–3* were amplified from inoculated adult plants only

[b]*H* and *M* refer to digestion with *Eco*RI + *Hpa*II and *Eco*RI + *Msp*I, respectively

Discussion

The MSAP has previously been applied to the study of genome methylation in various crops and was shown to be highly efficient for the large-scale detection of cytosine methylation (Xiong et al. 1999; Liu et al. 2001; Shaked et al. 2001; Madlung et al. 2002; Sherman and Talbert 2002). In this study, we have used the technique to study methylation of CCGG motifs in the rice genome at different developmental stages and following inoculation with the bacterial pathogen *X. oryzae* pv. *oryzae*. The cultivar studied here shows resistance to this pathogen at the mature stage (adult plant resistance); seedlings are susceptible.

Most of the changes observed in methylation (Class A) were associated with progression through development, and were not influenced by pathogen attack. A smaller set of sites (Class B) underwent demethylation in seedlings as a result of infection, and some other loci (Class D) were demethylated in adult plants upon infection (Table 2). Interestingly, no cases of post-infection hypermethylation were detected. These results suggest that the increase in the level of cytosine methylation during development may contribute to APR. Differences in the level of cytosine methylation among different organs or between different developmental stages have also been found in several other cases. In tomato, the level of DNA methylation was found to be higher in seeds than in mature leaves (Messeguer et al. 1991), and a higher level of DNA methylation was detected in seedlings than in flag leaves of rice (Xiong et al. 1999). In maize, the *Mu* transposable element normally undergoes hypermethylation in adult plants (Brown et al. 1994).

To further investigate whether methylation of functional genes is correlated with APR, all polymorphic fragments were sequenced, and six fragments were found to be homologous to functionally characterized genes (Table 3). These genes encode a Gag-Pol polyprotein,

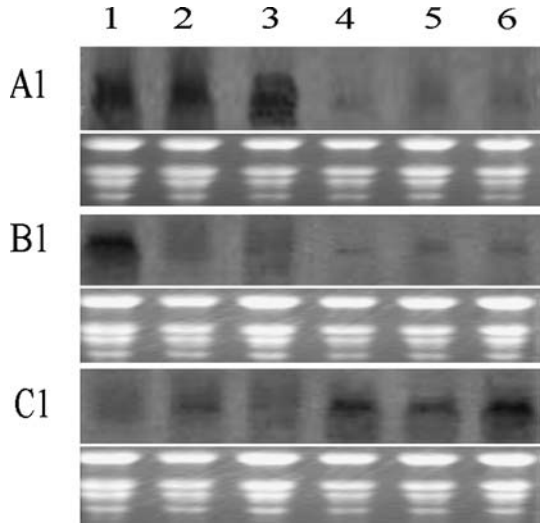

Fig. 2 Expression of the genes represented by fragments A1, B1, and C1 (see Table 3). Leaves from seedlings and adult plants inoculated with *X. oryzae* pv. *oryzae* strain T7133, mock-inoculated, or left untreated were harvested at 48 h for the isolation of total RNA, and Northern analysis was performed as described in Materials and methods. *Lanes 1 and 4* RNA from plants inoculated with the pathogen; *lanes 2 and 5* mock-inoculated plants; *lanes 3 and 6* untreated plants. *Lanes 1–3* show patterns from seedlings and *lanes 4–6* are from adult plants

the hypothetical protein PA2929, a homologue of CD8 antigen beta polypeptide, a beta-1, 4 endoglucanase, an RNA helicase of the Ski2 subfamily, and a receptor-like protein kinase, respectively. Gag-Pol polyproteins are encoded by the *gag-pol* genes of LTR (long terminal repeats)-retrotransposons and are responsible for the protease, reverse transcriptase, RNase H and integrase activities of LTR retrotransposons (Feschotte et al. 2002). Transcriptional activation of retrotranspons also has the potential to alter the expression of adjacent genes (Schramke and Allshire 2003; Kashkush et al. 2003). The CD8 antigen is a cell surface glycoprotein found on most cytotoxic T lymphocytes, and mediates efficient cell-cell interactions in the immune system (Zhang et al. 1996). β-1,4-Endoglucanases degrade cellulose in plant cell walls (Dalboege and Hansen 1994). The Ski2 subfamily of RNA helicases belongs to the DExH-box helicase family, which plays an essential role in the 3′ degradation of mRNA by the exosome complex (Colley et al. 2000). Receptor-like kinases (RLKs) have sequence homology and structural similarity with animal receptor kinases and are also thought to play important roles in signaling processes in plants also (Zhang, 1998). The methylation status of these genes is modified diffferentially in seedlings and adult plants upon pathogen attack (Table 3), suggesting that they may be involved in the APR response.

To investigate whether the modification of functional genes has any effect on their expression, three of them were analyzed by Northern hybridization. The results indicated that the expression of these genes in seedlings and adult plants was correlated with their methylation status (Fig. 2); as expected, hypomethylation was associated with increased expression.

With the exception of the gene for the putative receptor-like protein kinase, all six identified genes were methylated in adult plants (Table 3). Thus, this gene may play a crucial role in the APR response to infection with the bacterial blight pathogen.

In conclusion, significant differences in cytosine methylation are detected among the seedlings and adult plants of Wase Aikoku 3 by MSAP, and at least some of these may be related to the resistance of adult plants of this rice cultivar to bacterial blight. However, a much more extensive analysis will be required to confirm this.

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