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Full Length Research Paper

Tissue culture-induced alteration in cytosine methylation in new rice recombinant inbred lines

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Zizania **DNA introgression could induce a large number of genetic and epigenetic changes of the new rice recombinant inbred lines genome. In this present study, we employed inter-simple sequence repeat (ISSR) to further study the genetic and epigenetic changes that are induced by tissue culture. Changes induced by tissue culture are mostly epigenetic. One kind of methylation alterations is that the unmethylated CCGG sequence was transformed into external hemimethylated cytosines. This type change, however, did not transmit to progenies of the plant. Another kind of methylation alterations is tissue culture-induced occurrence of *de novo* methylation at the external cytosines of the CCGG sites that are originally with hyper-methylated internal cytosines. Interestingly, many of these methylated external cytosines became demethylated again upon plant regeneration, implying that the hypomethylated state of these loci is required for successful plant regeneration. Because the changed methylation patterns are highly similar among calli subcultured for different intervals, and because apparent concordance exists between randomly selected individual regenerants, we are tempted to deduce that the DNA methylation changes did not occur randomly, rather, the methylation events likely to have hot spots, and/or occur non-randomly. Sequence analysis indicates that the loci underwent methylation alterations are predominantly genic, sharing significant homology to known-function genes or expressed sequence tag (EST) sequences. This finding implies that the epigenetic changes induced by tissue culture could potentially affect the expression of the relevant genes.**

Key words: *Zizania latifolia*, rice, introgression line, genomic change, sequence change, epigenetic change, DNA methylation, ISSR.

INTRODUCTION

Plant tissue culture usually can induce genotypic and phenotypic variation. This phenomenon is called soma-clonal variation. It has been verified by the means of molecular markers in rice (Ngezahayo et al., 2007), cotton (Jin et al., 2008), rye (Linacero and Vazquez, 1993; Rakoczy-Trojanowska, 2002) and palm (Tregear et al., 2002; Kubis et al., 2003; Morcillo et al., 2006). It may also be an effective means of generating useful mutants, so it is used in plant breeding (Liu and Zheng, 2002; Heszky et al., 1989; Evans, 1989). DNA change is the main genetic variation induced by tissue culture. Recent studies have also found that tissue culture can induce DNA methylation variation, and thus affect the gene expression and even the phenotypic change (Miguel and Marum, 2011; Jaligot et al., 2002; Kaeppler et al., 2000). To explore the tissue culture induced DNA methylation variation and the genetic stability in their progenies is of great theoretical significance for understanding the epigenetic regulation mechanism and the influence on phenotype.

Introgression rice recombinant inbred lines are the new rice materials which are produced by a novel sexual

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Table 1. ISSR primers

Primer	Sequence (5'-3')	Primer	Sequence (5'-3')
4	BDB$(CA)_6$	21	GCGA$(CA)_6$
5	VHV$(GT)_7$	22	SSWN$(GACA)_3$
6	DBD$(GA)_7$	31	$(AG)_8$T
7	$(CT)_8$RG	32	$(AG)_8$C
9	$(CT)_8$RC	33	$(GA)_8$T
15	CCC$(GT)_6$	34	$(GA)_8$C
16	GSG$(GT)_6$	35	$(CT)_8$A
17	CSC$(GA)_6$	36	$(AG)_8$YT
18	GCW$(GA)_6$G	37	$(AG)_8$YC
20	CCAG$(TGG)_3$TG	39	$(CTTCA)_3$

R=A/G; Y=C/T; M=A/C; K=G/T; S=G/T; W=A/T; H=A/C/T; B=C/G/T; V=A/C/G; D=A/G/T; N=A/C/G/T.

hybridization approach between rice and wild rice (*Zizania latifolia*). A great deal of scientific works have confirmed that *Z. latifolia* DNA introgression into rice recombinant inbred lines results in a large number of DNA sequence variation (Wang et al., 2005), DNA methylation variation (Dong et al., 2006) and transposon tagging activation (Wang et al., 2010), and exhibited a wide range of phenotypic variations such as changes in over all plant statue, growth periods, yield components, and disease resistance, because of introgression, the introgression rice recombinant inbred lines are more sensitive and tends to be induced genetic variation and DNA methylation variation by tissue culture. This present study was aimed to provide some insight into the nature of somaclonal variation at the nucleotide sequence and DNA methylation level in introgression rice recombinant inbred lines. For this purpose, we performed multiple generations of rice tissue culture, and detected genomic variation and DNA methylation variation molecular markers technology, ISSR (inter-simple sequence repeat).

MATERIALS AND METHODS

Plant materials

The rice (cv. Matsumae), wild rice *Zizania latifolia* Griseb and two rice RILs, RZ1 and RZ35, were used in this study. The RILs were derived from intergeneric hybridization between rice (cv. Matsumae) and a local accession of wild rice *Z. latifolia* Griseb., into rice (*Oryza sativa* L.) by a (*Z. latifolia* Griseb.) novel sexual hybridization approach called "repeated pollination".

Plant tissue culture and ISSR (inter-simple sequence repeat) analysis

Sterilized mature seeds of rice were used in this study. Callus induction, maintenance and plant regeneration were performed according to an earlier report (Liu et al., 2004). After several months of subculture, portions of the calli were used for DNA extraction and subculture, and the others were used for regeneration. Rooted shoots were grown in a greenhouse before DNA extraction and further analysis.

ISSR amplifications were performed as reported previously (Guo et al., 2006), and the ISSR primers used in this study are listed in Table 1. Before PCR amplication, genomic DNA (~1.5µg) was digested with *HpaII* or *MspI* (New England Biolabs, Beverly, MA). ISSR amplification products were resolved on 2% agarose gels stained with ethidium bromide. Representative ISSR polymorphic bands were isolated and cloned for sequencing. The sequences were queried by BlastN and BlastX at the NCBI.

RESULTS

ISSR analysis of genome variation and epigenetic variation of introgression rice recombinant inbred lines materials

In order to identify the intermaterial differences in genome and DNA methylation state, the extracted DNA from three rice materials were divided into three groups: non digested, *Hpa*II degisted and MspI degisted, respectively. Then ISSR analyses were performed. Twenty ISSR primers obtained good amplification. The amplified fragments were 150 to 3,000 bp. Total 176 amplification sites were detected, in which many sequence variations and methylation variations were detected. The methylation variation was detected through the paired *Hpa*II and *Msp*I isoschizomers with different methylation sensitivity (Dong et al., 2006) (Table 2 and Figure 1).

ISSR analysis of genome variation and epigenetic variation of introgression rice recombinant inbred lines in the process of tissue culture

In all three rice materials, we detected a number of genomic variation and methylation variation. The performances included (1) external cytosines of CCGG sequence became (hemi)methylated. The CCGG sequence of seed-derived seedling was unmethylated. After tissue culture, various phase of callus appeared the (hemi) methylated cytosines, and the phenomenon

Table 2. ISSR results of non-tissue culture rice materials.

Model of different bands amplified by ISSR in introgression line		Number and percentage of site		
		non-digested	digested by *Hpa*II	digested by *Msp*I
Increase	Both RZ35 and RZ-2	6 (4.76%)	5 (3.70%)	3 (2.32%)
	RZ35	5 (3.97%)	8 (5.93%)	6 (4.65%)
	RZ-2	17 (13.5%)	18 (13.3%)	16 (12.4%)
Decrease	Both RZ35 and RZ-2	17 (13.5%)	11 (8.15%)	12 (9.30%)
	RZ35	4 (3.17%)	1 (0.74%)	6 (4.65%)
	RZ-2	16 (12.7%)	23 (17.0%)	25 (19.4%)

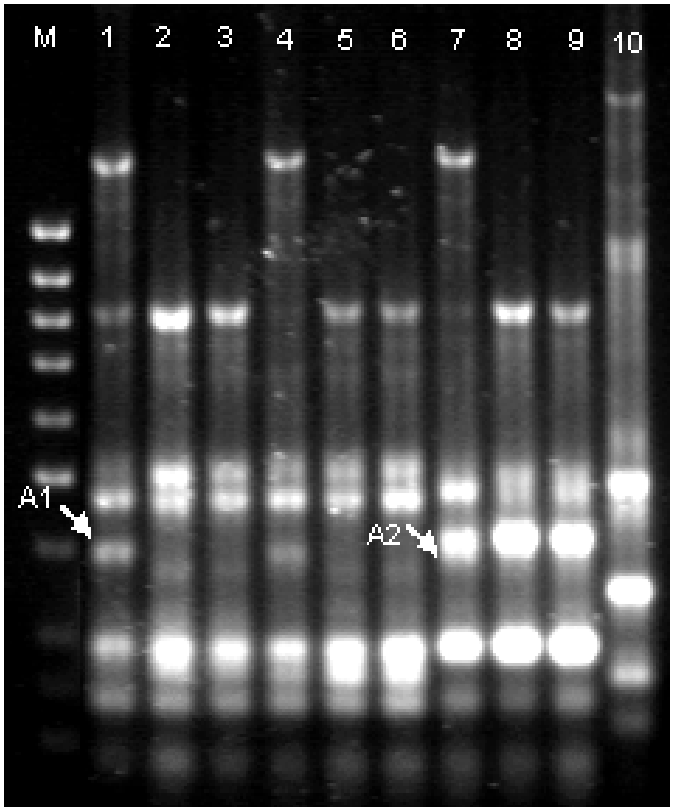

Figure 1. Examples of changing cytosine methylation patterns detected in the introgression lines (RZ2 and RZ35) as compared with their parent (Matsumae and *Zizania latifolia*) using ISSR. Lane M: marker; lanes 1~3: non degisted, Matsumae DNA degisted by *HpaII*, Matsumae DNA degisted by *MspI*; lanes 4~6: non degisted, RZ35 DNA degisted by *HpaII*, RZ35 DNA degisted by *MspI*; lanes 7~9: non degisted, RZ2 DNA degisted by *HpaII*, RZ2 DNA degisted by *MspI*; lane 10: *Zizania latifolia* DNA; A2 indicates methylation increase variation of introgression rice recombinant inbred lines.

was consistently disappeared in regeneration seedlings (Figures 2 and 4). This suggests that this kind of (hemi) methylation could not stably transmit to progenies through mitosis, and the methylation action only exists in callus. (2) External cytosines of CCGG sequence were tissue culture-induced occurrence of methylation. There were internal methylated cytosines of CCGG sequence in seed-derived seedling, and during tissue culture external cytosines were induced to be methylated Some external methylated cytosines were disappeared after differentiation and regeneration, and some can selectively stay in a portion of regeneration seedlings (Figures 3 and 4). 27

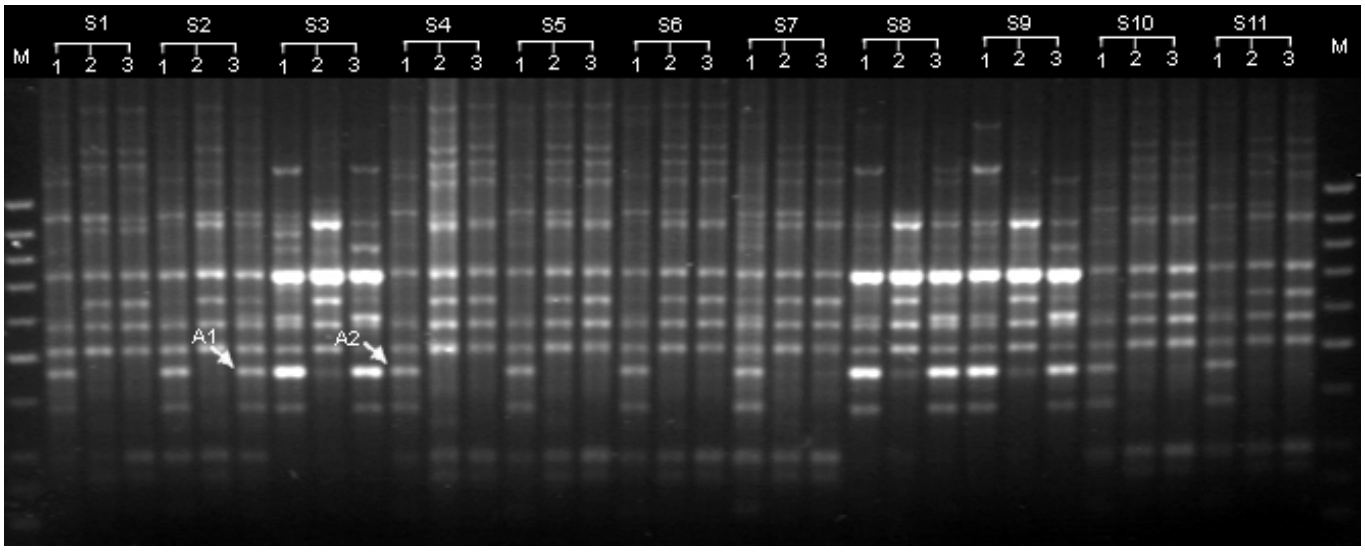

Figure 2. ISSR profiles displaying methylation variations in somaclones of rice cv. Matsumae. Lane M: Marker; lane 1: non degisted; lane 2: Matsumae DNA degisted by *HpaII*; lane 3: Matsumae DNA degisted by *MspI*. S1: non tissue culture materials; S2-S3: callus after 6 months; S4: regeneration seedlings induced from 3 months of callus; S5-S7: regeneration seedlings induced from 6 months of callus; S8-S9: callus after 12 months; S10-S11: regeneration seedlings induced from 12 months of callus. A1: hemimethylation variation in the process of tissue culture; A2: hemimethylation variation is disappeared in the regeneration seedlings.

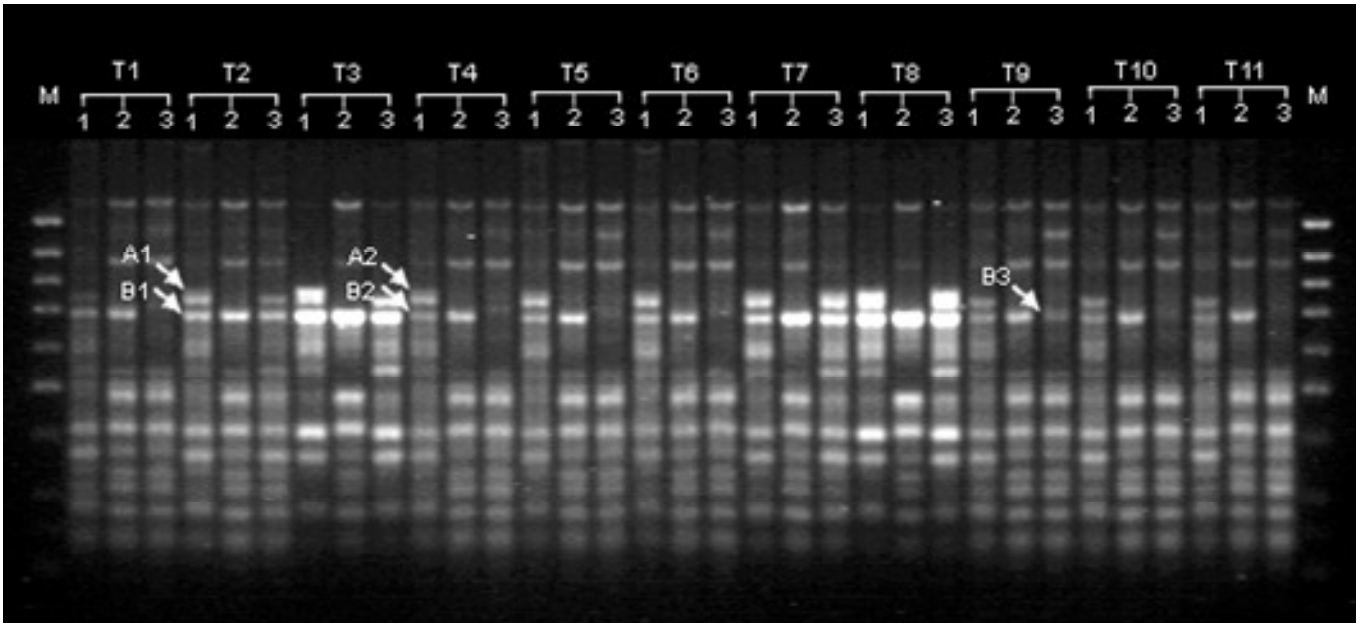

Figure 3. ISSR profiles displaying methylation variations in somaclones of introgression line RZ35. Lane M: Marker; lane 1: non degisted; lane 2: RZ35 DNA degisted by *HpaII*; lane 3: RZ35 DNA degisted by *MspI*. T1: non tissue culture materials; T2-T3: callus after 6 months; T4-T6: regeneration seedlings induced from 6 months of callus; T7-T8: callus after 12 months; T9-T11: regeneration seedlings induced from 12 months of callus. A1: hemimethylation phenomenon appeared in the process of tissue culture; A2: hemimethylation phenomenon disappearred in regeneration seedlings. B1: external methylated cytosines appeared in the process of tissue culture; B2: methylation increase phenomenon disappeared in 6 months regeneration seedlings; B3: methylation increase phenomenon remained in 12 months regeneration seedlings.

variation sequences were cloned and sequenced, of which 16 sequences were homology with known or unknown function gene, and accounted for 59.3% of all sequencing sequences. This demonstrates that the genome variation induced in the process of tissue culture is closely related with gene expression.

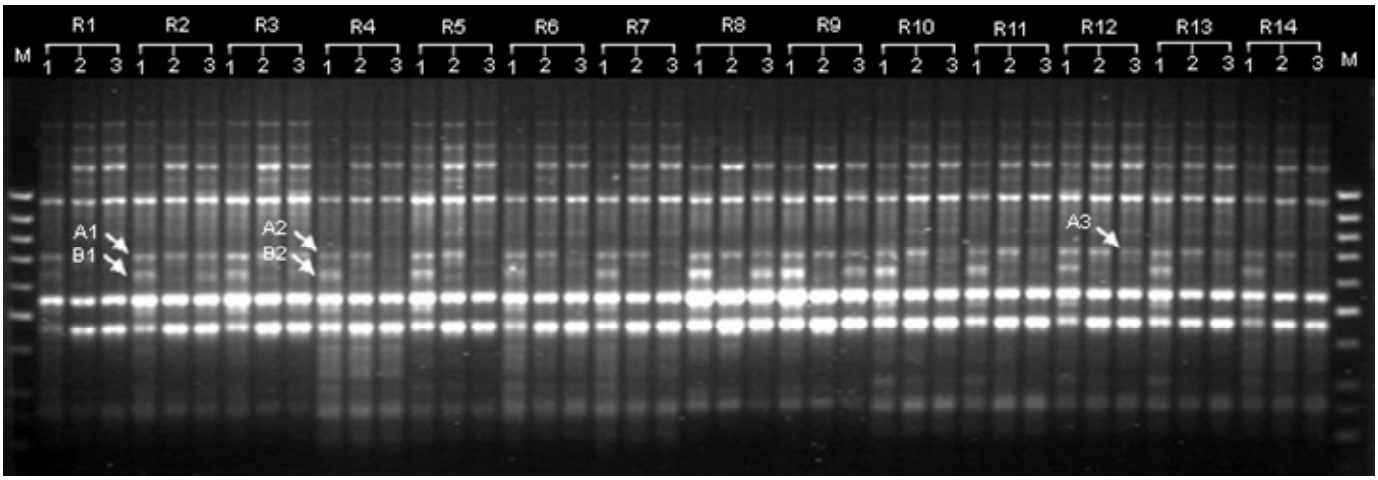

Figure 4. ISSR profiles displaying methylation variations in somaclones of introgression line RZ2. Lane M: Marker; lane 1: non degisted; lane 2: RZ2 DNA degisted by *HpaII*; lane 3: RZ2 DNA degisted by *MspI*. R1: non tissue culture materials; R2-R3: callus after 6 months; R4-R7: regeneration seedlings induced from 6 months of callus; R8-R9: callus after 12 months; R10-R13: induced from 12 months of callus; R14: regeneration seedlings induced from 15 months of callus. A1: external methylated cytosine appeared in the process of tissue culture; A2: methylation increase phenomenon disappeared in 6 months regeneration seedlings; A3: methylation increase phenomenon remained in 12 months regeneration seedlings; B1: hemimethylation phenomenon appeared in the process of tissue culture; B2: hemimethylation phenomenon disappeared in regeneration seedlings.

DISCUSSION

On (epi)genetic variation of introgression rice recom-binant inbred lines, previous study has demonstrated, at the molecular level that the progenies of introgression rice inbred lines do contain Zizania DNA (Wang et al., 2005). Epigenetic theory provides a possible explanation for extremely trace heterologous DNA introgression induces extensively genome variation of progenies. The introduction of Zizania DNA can induce extensive sequence variation, transposon tagging activation and methylation variation of introgression rice recombinant inbred lines genomes (Wang et al., 2005, 2010; Dong et al., 2006). This present study used ISSR molecular marker technology to explore the genome variation of introgression rice recombinant inbred lines. A large amount of sequence variations and methylation variations of CCGG sequence were also found. This suggests that the methylation pattern change may play an important role in introgression rice recom-binant inbred lines progenies formation. Meanwhile, introgression rice recombinant inbred lines progenies were detected in a large number of sequence dis-appearance and increase variations. The synthetic rape polyploid genomes research also found that there was random sequence disappearance and increase variation in progenies (Song et al., 1995). The mechanisms to occur phenotypic variation and DNA level variation in introgressive progenies have some similarities to the molecular mechanisms of genomic evolution in the process of alloploid speciation. According to this theory, the sequence disappearance and increase variation of introgression rice recombinant inbred lines also play an important role in the formation of introgression rice recombinant inbred lines. So, both methylation variations and sequence variations contribute to the formation of introgression rice recombinant inbred lines.

On tissue culture induced genome variation and epi-genetic variation, Kaeppler and Phillips (1993) proposed a hypothesis when studied DNA methylation changes happened in the process of maize tissue culture. They believed that DNA methylation changes may be a radical cause for somaclonal variation. In this present study, the detected callus genome variations were mainly methy-lation variations. According to the type of methylation, variations can be classified into the following two types: (1) the unmethylated CCGG sequence is transformed into external hemimethylated cytosines during tissue culture. This type change disappeared in, or in other words did not transmit to, progenies of the plant. (2) methylation variations are tissue culture-induced occur-rence of de novo methylation at the external cytosines of the CCGG sites that are originally with hyper-methylated internal cytosines. External cytosine methylation of some sites appeared the consistent disappearance in rege-neration seedlings. External cytosine methylation of some other sites can survive in a portion of regeneration seedlings. At the same time, the regeneration seedlings with the inheritance of methylation are the plants which differentiate from 1 year of callus. This suggests that the DNA methylation changes do not occur randomly; rather, the methylation variation sites are likely to have hot spots in the process of tissue culture.

Because there is no “detrimental” condition, that is, tissue culture, methylation state of many sites has also reversed. A portion of external cytosine methylations

can not transmit to the progenies, or steady transmit; the reason may be that the existing active demethylation mechanism in plant selectively remove the methyl of methylated cytosines. RdDM (RNA-directed DNA methylation) might be involved in this processing (Zhang and Zhu, 2011). And Chromamethyltransferase (CMT) and domains rearranged methyltransfease (DRM) should be required. Neverthless, the exact regulation mechanism is still unknown, which should be proved in further research.

DNA methylation patterns are highly variable in regeneration seedlings and progenies, showing that when compared with the seed-derived seedlings, DNA modification is more unstable in the regeneration plants from tissue culture. At the same time, to extending the time of tissue culture can remain some methylation variation, which transmits to the regeneration seedlings. This indicates that methylation variation accumulation and maintain are associated with tissue culture time (Kaeppler et al., 2000). This present study did not find a lot of methylation to be passed to the generation seedlings, and this may also be associated with tissue culture time. There may be active demethylation mechanism in plants to selectively remove a portion of callus double chain external methylated cytosine increase variation in regeneration seedlings.

Hemimethylation phenomenon is much common in tissue. External unmethylated cytosines were not methylated with the extension of time, still remained unmethylation state of CCGG sites, and happened the consistent reverse in the regeneration seedlings, implying that these sequences are associated with the gene expression change in the process of tissue culture. In addition, this also is associated with the selected materials. Because both RZ35 and RZ2 after all come from Matsumae, the three materials share the same genomic background in general.

We applied ISSR technology and successfully detected methylation variation in the course of tissue culture. Due to various single block callus and regeneration seedling, the detected methylation variation can not be mutations of a site or experimental pseudophase. ISSR can serve as a method for detecting DNA methylation variation.

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Supplementary Table 1. Blast X results of 27 sequenced bands.

Cloned band	Size (bp)	Primer	Homology and/or protein function predicted by BLAST X	Accession number	E-value
9-1	463	9			
9-2	409	9			
15-1	720	15	bHLH protein-like [Oryza sativa Japonica Group]	BAD46515.1	1.8e-05
15-2	640	15	bHLH protein-like [Oryza sativa Japonica Group]	BAD46515.1	0.00011
18-1	333	18			
20-1	900	20	unnamed protein product [Oryza sativa (japonica cultivar-group)]	NP_912917	2e-16
20-2	840	20	hypothetical protein [Oryza sativa Japonica Group]	BAD16259.1	3.3e-11
20-3	726	20	hypothetical protein [Oryza sativa Japonica Group]	BAB86106	4e-10
20-4	820	20	hypothetical protein [Oryza sativa Japonica Group]	AAS79731.1	2e-08
20-5	456	20			
21-1	750	21	putative PWWP domain protein [Oryza sativa Japonica Group]	BAD03546.1	1e-36
21-2	608	21	hypothetical protein [Oryza sativa Japonica Group]	NP_001046975.1	7.0e-42
21-3	423	21	hypothetical protein [Oryza sativa Japonica Group]	NP_001044077.1	1.1e-13
22-1	404	22			
31-1	1800	31	unknown protein [Oryza sativa Japonica Group]	BAC79522.1	8.9e-21
31-2	1600	31			
31-3	534	31			
32-1	575	32	hypothetical protein [Oryza sativa Japonica Group]	BAC98670.1	3e-18
32-2	547	32	hypothetical protein [Oryza sativa Japonica Group]	BAC79709.1	2.8e-07
33-1	625	33	phosphoglucomutase-like protein [Oryza sativa (japonica cultivar-group)]	BAC16110.1	3e-06
33-2	547	33			
34-1	500	34	putative nitrate-induced protein [Oryza sativa Japonica Group]	AAO37950.1	3.1e-50
36-1	1000	36	putative Acyl-CoA-binding protein [Oryza sativa Japonica Group]	BAD67905.1	0.00088
36-2	678	36			
36-3	672	36	putative secretory carrier membrane protein [Oryza sativa Japonica Group]	BAD07864.1	0.001
37-1	415	37			
37-2	409	37			