Identification of Simple Sequence Repeat Markers(SSR) Specific for Thermo-Sensitive Genic Male Sterile Rice Lines and Their Utilization in Assessment of Seed Purity

Numphet Sangarwut, Sriprapai Chakhonkaen, Keasinee Tongmark, Natjaree Panyawut, Kannika Sikaewtung, Thiwawan Wasinanon and Amorntip Muangprom*

The National Center for Genetic Engineering and Biotechnology (BIOTEC),

The National Science and Technology Development Agency (NSTDA)

Theerachai Thanananta

Department of Biotechnology, Faculty of Science and Technology, Thammasat University

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Abstract

Genetic purity is a key for hybrid rice seed production. Genetic purity of parental lines is an important factor affecting the genetic purity of the hybrid seeds. In this study, SSR markers specific for the thermo-sensitive genic male sterile (TGMS) rice lines were identified and used to assess their seed purity. A total of 40 SSR markers distributed on 12 rice chromosomes were used for genotyping 3 TGMS rice lines (IR68301-11-6-4-4-3-6-6, IR76761-4-3-17-34-35, and IR75589-31-27-8-33) obtained from international rice research institute (IRRI), and 20 Thai elite rice verities/lines. The results showed that RM341 was specific for IR68301-11-6-4-4-3-6-6. Four SSR markers (RM1359, RM1362, RM408, and RM219) and three SSR markers (RM190, RM225 and RM21) were specific for IR76761-4-3-17-34-35, and IR75589-31-27-8-33, respectively. In addition, SSR markers specific for eight Thai elite rice verities/lines were identified. The SSR markers specific for each TGMS line were used to determine their seed purity. Seed impurities were detected in 2 out of the 3 TGMS lines, suggesting the precaution for maternal seed production. These markers will be helpful for cultivar identification, seed purity test, intellectual property right (IPR), and future rice breeding programs.

Keywords: genetic purity; TGMS SSR markers; cultivar specific markers

1. Introduction

Rice (Oryza sativa L.) is an important global food crop. About half of the world population depends on rice. With the increasing of the world population and reduction of arable lands, there is an urgent need to increase rice yield to meet the global food demand (Bruinsma 2003, FAO 2012). In Thailand, rice is a major

*Corresponding author: amorntip.mua@biotec.or.th

food and a significant exported product. However, yield per hectare of Thai rice is less than that of neighboring countries such as Indonesia, Vietnam, Malaysia, Laos, and Philippines (USDA 2016).

Hybrid rice has been reported to increase yields by 20-30% higher than the best-inbred varieties in several countries (Yuan 1998; Virmani 2003; Akagi et al., 2004). The temperature-sensitive genic male-sterility (TGMS) system facilitates hybrid seed production (Maruyama et al. 1991; Wang et al., 2003). The expression of male sterility in TGMS lines is controlled by temperatures. Exposure to low or high temperatures at a specific developmental stage is essential for expressing sterility or fertility (Dong et al., 2000; Reddy et al., 2000). TGMS plants can be used as male-sterile lines for hybrid production, or self-propagation depending on temperature conditions, avoiding the need for maintainer lines. In addition, the hybrid system using TGMS enables the use of a wide range of genotypes as male parents, with no need for restorer lines. Furthermore, this system greatly reduces genetic vulnerability due to the restricted sterile cytoplasm because TGMS can be transferred to diverse genetic backgrounds, providing a broader genetic base of the hybrids and more choices for developing hybrids with desirable traits (Dong et al., 2000).

The success of hybrid technology depends mainly on the production of genetically pure hybrid seeds to ensure the gain of heterosis. It has been reported that 1% impurity in the hybrid seed reduces the yield of hybrid by about 100 kg/ha (Mao et al., 1996). Therefore, monitoring genetic purity at each stage of seed production is critical. Assessment of genetic purity is important for hybrid seed production. The genetic purity of hybrid seeds depends on the purity of parental lines and the processes for hybrid seed production, including pollen shedders and physical admixtures (Bora et al., 2016). Seed purity is conventionally determined by grow-out-test (GOT) depending on planting representative samples of those seeds and observing their phenotypes, which is time and resources consuming. In addition, several traits are affected by environmental conditions making variety identification difficult.

A molecular marker, based on DNA sequence variation, has been used to assess seed purity. Simple sequence repeat (SSR) markers are preferred to use for seed purity tests and cultivar identification due to their abundance, uniform distribution, and being co-dominant in nature (Ye-yun et al., 2005; Saxena et al., 2010; Sundaram et al., 2008; Bora et al., 2016). SSR has been used for genetic analysis, cultivar identification, and seed purity test in several crops, including rice (Ye-yun et al., 2005; Garris et al., 2005)

The present study aims to identify SSR markers specific for TGMS rice lines and use these markers to assess their seed purity. These markers will be useful for cultivar identification, intellectual property rights (IPR), and future rice breeding programs.

2. Methods

2.1 Plant materials

A total of 23 rice genotypes, including 3 TGMS obtained from the international rice research institute (IRRI) and 20 Thai elite lines potentially could be used as hybrid parents were used in this study (Table1). For polymorphism test and identification of cultivar specific markers, genomic DNA of the 23 rice genotypes was isolated from bulked leaves of 2-3 week old seedlings using 10 plants for each genotype. For assessment of seed purity of the 3 TGMS lines, genomic DNA was isolated from leaves of 2-3 week old single seedlings with 40 seedling/ genotype. In addition, the DNA of representative rice lines having polymorphic DNA patterns was included.

2.2 Molecular analysis

Genomic DNA was isolated using Cetyl-Trimethyl Ammonium Bromide (CTAB) method (Murray and Thompson 1980), and quantified using Nanodrop (NanoDrop ND. 1000, Thermo Scientific, USA). Forty SSR markers (Table 2) were selected from 12 rice chromosomes (Garris *et al*. 2005). The genomic DNA samples were subjected to PCR amplification using SSR markers as primers. The PCR amplification was performed in 10 µL of the reaction mixture, containing 15 ng genomic DNA, 1× *Taq* DNA polymerase buffer, 1. 5 mmol/ L $MgCl_2$, 0. 2 mmol/ L dNTPs, 0. 25 U *Taq* DNA polymerase (Invitrogen, USA) and 1 µl (10 µmol/ L) of each primer. Thermal profiling was set up with an initial denaturation temperature of 95 ºC for 5 min followed by 35 cycles of denaturation (95 ºC for 45 s), annealing (50- 67 ºC for 45 s), and extension (72 ºC for 1 min), and a final extension (72 ºC for 8 min). PCR products (3 µL) were quality checked on 1. 5% agarose gel electrophoresis. The amplified PCR products together with a 100 bp DNA ladder (Thermo Scientific, USA) were then size separated on the 8% vertical polyacrylamide gel electrophoresis, and DNA patterns were observed by silver staining (Bassam *et al.*, 1991; Panaud *et al.*, 1996). The resulting markers specific for each TGMS line were used to test seed purity of that TGMS rice lines using 2 sets of random samples of 20 seeds each. A total of at least 40 seeds were tested for each genotype. The impurities were identified based on deviation in the expected amplification pattern.

3. Results and Discussion

3. 1 Identification of cultivar specific markers

Genotyping using 40 SSR markers tested with 23 rice genotypes showed that there were 3 patterns of DNA bands gained from the PCR products: monomorphic, polymorphic but not specific, and polymorphic and specific for a rice genotype. The results showed that RM341 was specific for IR68301-11-6-4-4-3-6-6 (No.15, Fig 1A). This marker was able to distinguish IR68301-11-6-4-4-3-6-6 from the other 2 TGMS lines (IR76761- 4- 3- 17- 34- 35, No. 16 and IR75589- 31- 27- 8- 33, No. 17) and the other tested Thai elite lines.

Table 1 Rice genotypes used in this study.

No.	Names	Traits
1	KLG96006-76-1-1-1-3	High yield, High grain quality, Resistant to Blast and BPH
2	SPR93014-PTT-22-1-3-2-1	High yield, Moderately resistant to Blast, Blight, and BPH
3	SPR90	High yield, High grain quality, Resistant to Blast and BPH
4	SPR93039-PTT-4-2-1-2-2-1	High yield, Moderately resistant to Blast, Blight, and BPH
5	PSL03450-91-1-6-1	High yield, High grain quality, Resistant to Blast and BPH
6	KLG02008-19-2-2-4-3	High yield, High grain quality, Resistant to Blast and BPH
7	RD 39	High cooking quality, Resistant to Blast and Blight
8	RD41	High yield, High grain quality, Resistant to Blast and BPH
9	SPR 1	High yield, Resistant to Blast, Blight, and BPH
10	RD 31	High yield, Resistant to Blast, Blight, and BPH
11	RD29	High yield, High grain quality, Resistant to BPH, Early flowering
12	PTT98044-64-1-1-2-1	High yield, High grain quality, Resistant to Blast and BPH
13	CNT96013-5-1-PSL-2-4-3	High yield, Resistant to Blast, Blight, and BPH
14	CNT96024-61-1-PSL-1-2	High yield, High grain quality, Resistant to Blast and BPH
15	IR68301-11-6-4-4-3-6-6(B2)	Thermo-sensitive genic male sterile: TGMS
16	IR76761-4-3-17-34-35(B6)	Thermo-sensitive genic male sterile: TGMS
17	IR75589-31-27-8-33(B8)	Thermo-sensitive genic male sterile: TGMS
18	PTT1	High yield, Fragrance, High cooking quality, Resistant to BPH, Blast and Blight
19	PSL2	High yield, High grain quality, Resistant to BPH
20	SPR 91062-5-PTT-1-2-1	High yield, Moderately resistant to BPH
21	PSL 60-2	High yield, Moderately resistant to BPH, Blast and Blight
22	Khomchonlasit	High cooking quality, Resistant to flooding, Resistant to lodging
23	Pin 3	High yield, Fragrance, High amylose content

Table 2 Details of SSR markers used in this study

No	SSR markers	Chr.	Forward primer 5'-3'	Reverse primer 5'-3'	Annealing Temp
1	RM1	1	GCGAAAACACAATGCAAAAA	GCGTTGGTTGGACCTGAC	55°C
2	RM237	1	CAAATCCCGACTGCTGTCC	TGGGAAGAGAGCACTACAGC	55°C
3	RM431	1	TCCTGCGAACTGAAGAGTTG	AGAGCAAAACCCTGGTTCAC	55°C
4	RM495	1	AATCCAAGGTGCAGAGATGG	CAACGATGACGAACACAACC	55°C
5	RM112	2	GGGAGGAGAGGCAAGCGGAGAG	AGCCGGTGCAGTGGACGGTGAC	55°C
6	RM279	2	GCGGGAGAGGGATCTCCT	GGCTAGGAGTTAACCTCGCG	60°C
7	RM341	2	CAAGAAACCTCAATCCGAGC	CTCCTCCCGATCCCAATC	57°C
8	RM282	3	CTGTGTCGAAAGGCTGCAC	CAGTCCTGTGTTGCAGCAAG	57°C
9	RM338	3	CACAGGAGCAGGAGAAGAGC	GGCAAACCGATCACTCAGTC	55°C
10	RM514	3	AGATTGATCTCCCATTCCCC	CACGAGCATATTACTAGTGG	55°C
11	OSR13	3	CATTTGTGCGTCACGGAGTA	AGCCACAGCGCCCATCTCTC	55°C
12	RM142	4	CTCGCTATCGCCATCGCCATCG	TCGAGCCATCGCTGGATGGAGG	57°C
13	RM317	4	CATACTTACCAGTTCACCGCC	CTGGAGAGTGTCAGCTAGTTGA	57°C
14	RM1359	4	CTCGCGAGGAAGAAGACAAC	CGCCGGCTGGTTAATTAATC	55°C
15	RM122	5	GAGTCGATGTAATGTCATCAGTGC	GAAGGAGGTATCGCTTTGTTGGAC	55°C
16	RM13	5	TCCAACATGGCAAGAGAGAG	GGTGGCATTCGATTCCAG	55°C
17	RM161	5	TGCAGATGAGAAGCGGCGCCTC	TGTGTCATCAGACGGCGCTCCG	67°C
18	RM437	5	ACACCAACCAGATCAGGGAG	TGCTCGTCAATGGTGAGTTC	59°C
19	RM190	6	CTTTGTCTATCTCAAGACAC	TTGCAGATGTTCTTCCTGATG	57°C
20	RM225	6	TGCCCATATGGTCTGGATG	GAAAGTGGATCAGGAAGGC	57°C
21	RM3431	6	ATCCAAATCCAATGGTGC	GCGAAAGGGAACATTCTG	57°C
22	RM1362	7	TGATCTAAACAGGCCCTTAG	CATCATCAAGACCACACATC	57°C
23	RM214	7	CTGATGATAGAAACCTCTTCTC	AAGAACAGCTGACTTCACAA	57°C
24	RM455	7	AACAACCCACCACCTGTCTC	AGAAGGAAAAGGGCTCGATC	59°C
25	RM152	8	GAAACCACCACACCTCACCG	CCGTAGACCTTCTTGAAGTAG	55°C

Table 2 (Continue) Details of SSR markers used in this study

No.	SSR markers	Chr.	Forward primer 5'-3'	Reverse primer 5'-3'	Annealing Temp.
27	RM408	8	CAACGAGCTAACTTCCGTCC	ACTGCTACTTGGGTAGCTGACC	55°C
28	RM105	9	GTCGTCGACCCATCGGAGCCAC	TGGTCGAGGTGGGGATCGGGTC	59°C
29	RM215	9	CAAAATGGAGCAGCAAGAGC	TGAGCACCTCCTTCTCTGTAG	59°C
30	RM219	9	CGTCGGATGATGTAAAGCCT	CATATCGGCATTCGCCTG	57°C
31	RM316	9	CTAGTTGGGCATACGATGGC	ACGCTTATATGTTACGTCAAC	50°C
32	RM147	10	TACGGCTTCGGCGGCTGATTCC	CCCCCGAATCCCATCGAAACCC	57°C
33	RM271	10	TCAGATCTACAATTCCATCC	TCGGTGAGACCTAGAGAGCC	50°C
34	RM228	10	CTGGCCATTAGTCCTTGG	GCTTGCGGCTCTGCTTAC	57°C
35	RM144	11	TGCCCTGGCGCAAATTTGATCC	GCTAGAGGAGATCAGATGGTAGTGCATG	57°C
36	RM181	11	ACGGGAGCTTCTCCGACAGCGC	TATGCTTTTGCCGTGTGCCGCG	57°C
37	RM21	11	ACAGTATTCCGTAGGCACGG	GCTCCATGAGGGTGGTAGAG	57°C
38	RM17	12	TGCCCTGTTATTTTCTTCTCTC	GGTGATCCTTTCCCATTTCA	57°C
39	RM270	12	GGCCGTTGGTTCTAAAATC	TGCGCAGTATCATCGGCGAG	57°C
40	RM512	12	CTGCCTTTCTTACCCCCTTC	AACCCCTCGCTGGATTCTAG	55°C

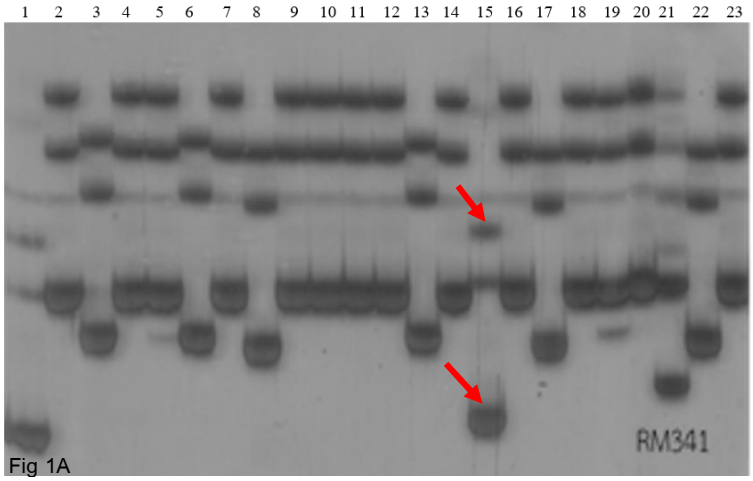
In addition, RM341 was specific for several Thai elite lines such as KLG96006-76-1-1-1-3, No.1 and PSL60-2,No. 21. RM1359, RM1362, RM408 and RM219 were specific for IR76761-4-3-17-34-35, No. 16. The results of genotyping using RM1359 was shown in Fig 1B. RM190, RM225 and RM21 were specific for IR75589-31-27-8-33. In addition, the results showed several SSR markers specific for 8 Thai elite lines (Table 3). Furthermore, the results indicated that some SSR markers such as RM1362, RM341, RM219, and RM1359 were highly polymorphic (Table3).

3.2 Seed purity tests

The resulting TGMS specific markers were used to determine the seed purity of each TGMS rice line using at least 40 seedlings for each line. The results showed that no contaminant was detected in IR68301-11-6-4-4-3-6-6 using RM341 (Fig 2). However, 3 contaminants were detected in IR76761-4-3-17-34-35 using RM408 (Fig 3) and 3 contaminants were detected in IR75589-31-27-8-33 using RM21 (Fig 4). These results suggested that the seed lots of IR68301-11-6-4-4-3-6-6 had 100% seed purity, while IR76761-4-3-17-34-35 and IR75589-31-27-8-33 had 92.5% seed purity.

Table 3 SSR Markers identified to be specific for some rice genotypes.

No.	Rice genotypes	Specific SSR Markers
1	KLG96006-76-1-1-1-3	RM341, RM1359, RM455 and RM271
2	SPR93014-PTT-22-1-3-2-1	-
3	SPR90	RM1362
4	SPR93039-PTT-4-2-1-2-2-1	-
5	PSL03450-91-1-6-1	-
6	KLG02008-19-2-2-4-3	-
7	RD39	RM310
8	RD41	-
9	SPR1	-
10	RD31	-
11	RD29	RM1362
12	PTT98044-64-1-1-2-1	-
13	CNT96013-5-1-PSL-2-4-3	RM1362
14	CNT96024-61-1-PSL-1-2	-
15	IR68301-11-6-4-4-3-6-6	RM341
16	IR76761-4-3-17-34-35	RM1359, RM1362, RM408 and RM219
17	IR75589-31-27-8-33	RM190, RM225 and RM21
18	PTT1	RM237 and RM219
19	PSL2	-
20	SPR 91062-5-PTT-1-2-1	RM20
21	PSL 60-2	RM1, RM237, RM341, and RM219
22	Homchonlasit	RM1, RM310, and RM271
23	Pin 3	RM144



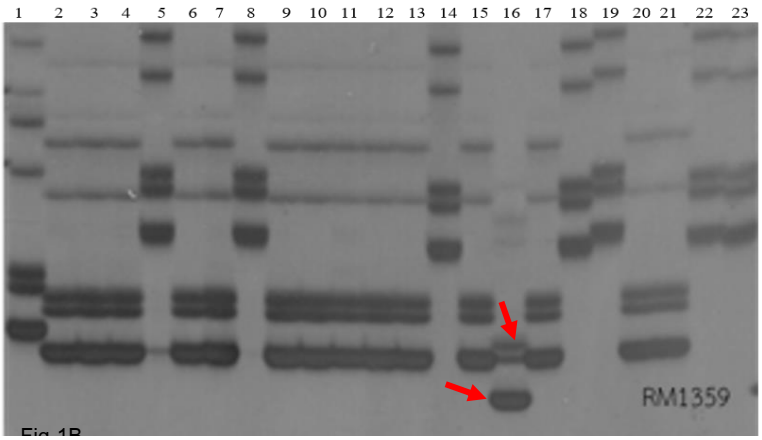


Figure 1 Cultivar specific markers: A, RM341 specific for TGMS IR68301-11-6-4-4-3-6-6(B2), and some Thai elite lines; B, RM1359 specific for IR76761-4-3-17-34-35(B6) and some Thai elite lines. Each lane contained bulked DNA of 10 seedling of each rice line. Lane 1-23 are rice lines; 1= KLG96006-76-1-1-1-3, 2= SPR93014-PTT-22-1-3-2-1, 3=SPR90, 4= SPR93039-PTT-4-2-1-2-2-1, 5= PSL03450-91-1-6-1, 6= KLG02008-19-2-2-4-3, 7= RD39, 8= RD41, 9= SPR1, 10= RD31, 11= RD29, 12= PTT98044-64-1-1-2-1, 13= CNT96013-5-1-PSL-2-4-3, 14= CNT96024-61-1-PSL-1-2, 15= IR68301-11-6-4-4-3-6-6(B2), 16= IR76761-4-3-17-34-35(B6), 17=IR75589-31-27-8-33(B8), 18= PTT1, 19= PSL2, 20= SPR 91062-5-PTT 1-2-1, 21= PSL60-2, 22= Homchonlasit and 23= Pin3. Arrows indicate the specific bands.

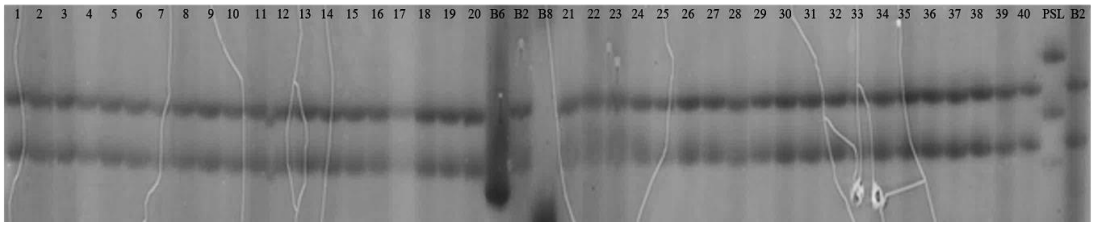


Figure 2 Genetic purity of TGMS IR68301-11-6-4-4-3-6-6 (B2) using RM341. No. 1-40 = individual plants representing a random sample from seed lot of TGMS IR68301-11-6-4-4-3-6-6(B2). B6= IR76761-4-3-17-34-35, B2= IR68301-11-6-4-4-3-6-6, B8= IR75589-31-27-8-33 and PSL = PSL2

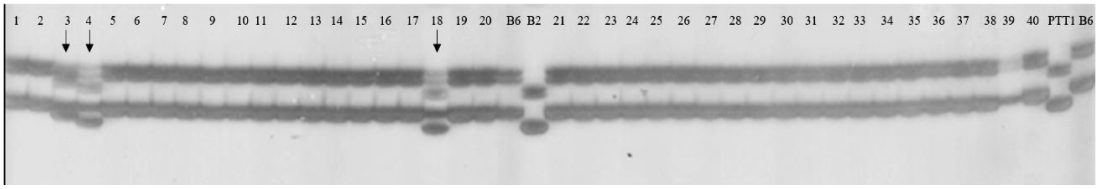


Figure 3 Genetic purity of TGMS IR76761-4-3-17-34-35(B6) using RM408. No. 1-40 = individual plants representing a random sample from seed lot of TGMS IR76761-4-3-17-34-35. B6= IR76761-4-3-17-34-35, B2= IR68301-11-6-4-4-3-6-6 and PTT1; Arrows represent the off-type/contaminant.

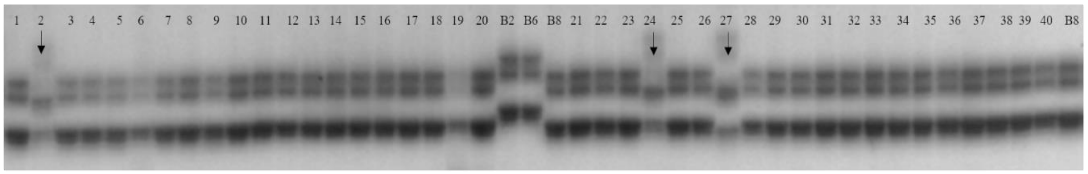


Figure 4 Genetic purity of TGMS IR75589-31-27-8-33 (B8) using RM21. No. 1-40 = individual plants representing a random sample from seed lot of TGMS IR75589-31-27-8-33 B6= IR76761-4-3-17-34-35, B2= IR68301-11-6-4-4-3-6-6 and B8= IR75589-31-27-8-33; Arrows represent the off-type/contaminant

4. Discussion

SSR markers have been used for cultivar identification and seed purity assessment in several crops such as rice (Ye-yun *et al*., 2005), sunflower (Pallavi *et al*., 2011), maize (Wu *et al*., 2006), and pigeon pea (Saxena *et al*., 2010). Several studies reported that the use of SSR markers for seed purity test are comparable with grow-out-test (GOT) (Wu *et al*., 2006; Bora *et al*., 2016; Kumar *et al*., 2012). The GOT is based on morphological traits, which are affected by the environment, making determination difficult. In addition, many modern cultivars are phenotypically less distinct, making morphological evaluation more difficult. Furthermore, GOT is time-consuming, tedious, costly, and excessive use of land (Noli *et al*.,

1999; Sundaram *et al.*, 2008). SSR marker, in contrast, based on DNA sequence variation, provides an unbiased means of crop identification. In addition, SSR markers being co-dominant in nature are preferred as desirable markers in rice, besides their abundance and uniform distribution in the rice genome (Akagi *et al*., 1996; McCouch *et al*., 1997).

Similar to other studies, we assessed the potential use of SSR markers in distinguishing rice varieties and utilized the resulting cultivar specific markers to assess seed purity (Sundaram *et al*., 2008; Bora *et al*., 2016; Kumar *et al*., 2012; Nandakumar *et al*., 2004). We were able to identify SSR markers specific for all the 3 TGMS male sterile lines potentially used as maternal lines for 2 line-hybrid production. These markers distinguished the 3 TGMS lines from each other, and the other tested rice lines were potentially used as parental lines for 2 line-hybrid production. The number of SSR markers specific for each TGMS line was different. Only one SSR marker was identified as a specific marker to IR68301-11-6-4-4-3-6-6, while 4 and 3 markers were identified as specific markers for IR76761-4-3-17-34-35 and IR75589-31-27-8-33, respectively. The results suggested that IR68301-11-6-4-4-3-6-6 probably has genetic background that is more similar to the other tested rice line than the other 2 TGMS lines. In addition, we were able to identify SSR markers specific for 8 out of 20 tested Thai elite lines. Similarly, the number of SSR markers specific for each Thai elite line were different. The results showed that KLG96006-76-1-1-1-3 and PSL60-2 have the highest number of SSR specific markers suggesting that they are more distinct from the other tested Thai elite lines. Although these SSR markers were not linked or a part of any functional genes, these cultivar specific markers will be useful for variety authentication, registration, seed purity test, and plant variety protection. Assessment of seed purity is very important for hybrid seed production. Seed purity of parental line is one of the major factors affecting purity of hybrid seeds. In two-line hybrid, purity of maternal male sterile line could be affected by cross-pollination and mechanical mixture. Using a cultivar specific SSR marker, we detected contaminants in our TGMS seeds. We did not confirm our results with GOT due to other studies already reported that using SSR marker for seed purity test is comparable with GOT (Bora *et al.*, 2016; Nandakumar *et al.*, 2004; Kumar *et al.*, 2012). Bora *et al.*, (2016) and Nandakumar *et al.*, (2004) reported that genetic purity tested by a single SSR marker with 50 random hybrid seeds was comparable with GOT in the field using 400 seeds from the same seed lot. In addition, Kumar *et al*., (2012) validated that using a single SSR marker with 30 random hybrid seeds was comparable with GOT in the field using 400 seeds from the same seed lot.

This present study is our preliminary results for our two-line-hybrid development. Our results suggested that the production of maternal male-sterile seeds should be aware of the contamination.

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