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Rapid method for detecting SNPs on agarose gels and its application in candidate gene mapping

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Abstract TILLING (Targeting Induced Local Lesions IN Genomes) exploits the fact that CEL I endonuclease cleaves heteroduplexes at positions of single nucleotide or small indel mismatches. To detect single nucleotide polymorphisms (SNPs) across a population, DNA pools are created and a target locus under query is PCR-amplified and subjected to heteroduplex formation, followed by CEL I cleavage. Currently, the common method used to detect cleaved products is by polyacrylamide gel electrophoresis using a high-throughput genotyping platform. Exact SNPs are then determined by sequencing. We sought to simplify the detection of CEL I-cleaved products on conventional agarose gels to make the technique more accessible to collaborating partners in developing countries where access to instrumentation could be limiting. Here, we used a panel of stress-related genes to evaluate SNP detection across 48 rice genotypes by contrasting them individually against IR64 and Nipponbare. SNP detection calls corresponded perfectly with those obtained from the Li-Cor genotypers. We were able to detect SNPs in pools of eight DNA templates, suggesting that the agarose gel system could be used to screen for SNPs with comparable throughput as that of the Li-Cor genotypers and showed that the throughput can be increased by analyzing larger amplicons (~3 kb). The agarose method offers a significant advantage by alleviating the need for labeled primers. We further demonstrated that the agarose method can be effectively used in gene mapping, an application particularly useful for parental lines with low levels of polymorphism. The lower cost and simplicity of the technique make it possible for broader applications of SNP-based markers for germplasm characterization and mapping studies.

Keywords TILLING/EcoTILLING · SNP detection · CEL I · Agarose · Mapping · Rice

Abbreviations

TILLING	Targeting induced local lesions IN genomes
SNP	Single nucleotide polymorphism

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TPP	Trehalose 6-phosphate phosphatase
ADF	Actin depolymerizing factor
RILs	Recombinant inbred lines

Introduction

Single-base changes and small insertions and deletions (indels) are the most common forms of genetic variation in natural populations, reflecting the results of evolution and adaptation(Yamanaka et al. 2004; Wright et al. 2005). Identifying single-nucleotide polymorphisms (SNPs) and indels across germplasm is of key interest to researchers involved in crop genetic studies (Syvanen 2001; Rafalski 2002; Borevitz et al. 2003). Further, the expanding amount of sequence data in plants has made it possible to apply reverse genetic tools to identify gene function (Henikoff and Comai 2003; Till et al. 2004). TILLING (Targeting Induced Local Lesions IN Genomes) and its derivative EcoTILLING were developed as techniques to identify SNPs in a population of mutants and ecotypes, respectively (McCallum et al. 2000; Comai et al. 2004). An EcoTILLING/TILLING experiment typically involves the following steps:

1. DNA extraction, quantification and normalization.
2. Pooling of DNA.
3. PCR amplification of genomic region under query.
4. Heteroduplex formation.
5. Cleavage using CEL I, an endonuclease.
6. Detection of cleaved products.
7. Sequencing of full length PCR products.

The DNA pools are used as templates to amplify the genomic region of interest. For the purpose of mutant discovery or TILLING, commonly DNAs from eight samples are pooled whereas, for EcoTILLING, DNAs from two genotypes are combined. This difference in pooling is due to the fact that more SNPs are expected between distant genotypes while the occurrence of SNPs in a mutant population is far lower.

The PCR products are then subjected to conditions wherein heteroduplexes are likely to form due to SNPs. The heteroduplexes are then cleaved using an endonuclease extracted from celery called CEL I which cleaves heteroduplexes at positions of mismatch (Oleykowski et al. 1998). Post-cleavage products are most commonly separated on polyacrylamide gels and visualized using laser technology.

Currently, most TILLING experiments use polyacrylamide gel electrophoresis and a high throughput genotyping platform for detecting CEL I-cleaved products (Colbert et al. 2001; Comai et al. 2004; Henikoff et al. 2004; Slade et al. 2005). This method requires the use of fluorescently labeled primers (specifically labeled with IRDye 700 and IRDye 800) and software for image analysis (Comai et al. 2004). In another study, a 10% polyacrylamide gel was used to separate the CEL I-cleaved products followed by staining with SYBR green (Yang et al. 2004). Other techniques employed to separate CEL I-cleaved products include the ABI 377 sequencer (Perry et al. 2003) and the Transgenomic WAVE-HS system (Caldwell et al. 2004). Suzuki et al. (2005) demonstrated the use of capillary electrophoresis on the HDA-GT12 system of eGene Inc. (USA) to separate CEL I-cleaved products to screen for rice mutants.

Previous studies have used agarose gels to separate CEL I-cleaved fragments of PCR products from bacteria, plasmids and human mitochondrial genome(Sokurenko et al. 2001; Bannwarth et al. 2005). Greber et al. (2005) compared different PCR-based methods of mutation detection in embryonic stem cell clones and discussed the use of agarose gels as a cost effective method. Preliminary experiments suggest that rice is amenable for TILLING (Wu et al. 2005); however, having a low-cost and simplified technique is important for many rice researchers in developing countries where access to instrumentation can be limiting.

This study sought to simplify TILLING by developing a robust procedure for detecting CEL I-cleaved products on conventional agarose gels. We evaluated the level of pooling and the size of genomic fragments that can be scanned, and compared the results of agarose with those using

the standard TILLING procedure based on the Li-Cor genotyper. In addition, we extended the agarose SNP detection method to map candidate genes in a segregating population. Our results showed that the SNP detection calls corresponded perfectly with those based on Li-Cor genotyper. We confirmed that it was possible to detect SNPs in an eightfold pool of DNA samples (or detecting 1 in 15 alleles, assuming the variant is in a heterozygous state) and were able to scan amplicons as large as 3 kb. Although the agarose method lacks the resolution to define SNP haplotypes, it offers a significant advantage by alleviating the need for labeled primers. We further showed that SNP detection on agarose can be an efficient method to map candidate genes from parental lines that show low levels of polymorphism. The lower cost and simplicity of the technique make it possible for broader applications of SNP-based markers for germplasm characterization and mapping studies.

Materials and methods

Plant materials and DNA pooling

A set of 48 *O. sativa* accessions (Table 1) were obtained from either the INGER—the International Network for the Genetic Evaluation of Rice (IRTP accessions) or the International Rice Genebank (IRGC accessions). Sample DNAs were extracted from 200 mg leaf tissue using the method described by Fulton et al. (1995). DNAs from all samples were quantified spectrophotometrically or by gel densitometry against a low mass DNA ladder and normalized to a concentration of 0.5 ng/μl. For EcoTILLING, DNA from each genotype was contrasted with IR64 and Nipponbare separately in a 1:1 ratio. Since rice is an inbreeding species, most loci are assumed to be homozygous; however, to ensure that mismatches were not caused by heterozygosity, DNAs from individual plants were assayed as controls in all cases.

To test the optimum pool size for the purpose of TILLING, we combined the DNA from known mutants of IR64 and wild type IR64 at ratios 0:1, 1:0, 1:1; 1:3, 1:7, 1:11, 1:15, 1:19, 1:23, 1:27, 1:31, 1:35, 1:39 prior to PCR amplification of the locus for oxalate oxidase-like protein on chromosome 8 (LOC_Os08g09010). DNAs from a mutant and a selected progeny from this mutant, M715, a M_2 line known to be heterozygous at this locus and M715-1, a homozygous M_3 line selected from M715, were combined with wild type DNA to test the sensitivity of detecting homozygous versus heterozygous alleles among M_2 plants.

Gene loci and primers

Genes used in this study were MYB1 on chromosome 12, trehalose 6-phosphate phosphatase (TPP) on chromosome 2, actin depolymerizing factor (ADF) on chromosome 2, and two oxalate oxidase genes and an oxalate oxidase-like gene on chromosome 8. The TIGR locus IDs and primers used in this study are listed in Table 2. For TILLING or EcoTILLING on the Li-Cor, forward primers 5′-labeled with IRDye 700 and reverse primers 5′-labeled with IRDye 800 were obtained from Metabion, GmbH (Martinsried, Germany; http://www.metabion.com). Unlabeled primers for agarose based assays were obtained from either Metabion or Invitrogen, Inc. (Hong Kong, China; http://www.invitrogen.com).

EcoTILLING on Li-Cor genotyper

The PCRs were performed in 10 μl final volume on 2.5 ng DNA (pooled and un-pooled) using 0.4 U/reaction of *Taq* DNA polymerase (TaKaRa Ex Taq™). A cocktail of labeled and unlabeled forward and reverse primers was used for PCR amplification. The mixture contained a 3:2 ratio of IRD700-labeled to unlabeled forward primers and 4:1 ratio of IRD800-labeled to unlabeled reverse primers. The addition of primers and subsequent steps were carried out under minimal light since the fluorescent labels are photosensitive. The PCR was performed as follows: one cycle at 95°C for 2 min; 35 cycles at 94°C for 20 s, 68°C for 30 s and 72°C for 30 s and one final cycle at 72°C for 5 min. The PCR products were denatured at 99°C for 10 min and renatured initially at 70°C for 20 s followed by 69 cycles with the temperature decreased by 0.3°C per cycle.

Table 1 Germplasm varieties for EcoTILLING

Code	Variety name	Accession	Code	Variety name	Accession
1	CT9993-5-10-1-M	IRTP 18527	25	Jagli Boro	IRGC 27516
2	Apo (IR55423-01)	IRTP 19122	26	Khao Dawk Mali 105	IRGC 27748
3	Vandana	IRTP 19187	27	Leuang Pratew	IRGC 27762
4	IR62266-42-6-2	IRTP 18979	28	Rathal	IRGC 31524
5	IR60080-46A	IRTP 21016	29	Gerdeh	IRGC 32301
6	Dee-Geo-Woo-Gen	IRGC 123	30	Tchampa	IRGC 32368
7	Azucena	IRGC 328	31	Dular	IRGC 32561
8	Carolina Gold	IRGC 1723	32	Peta	IRGC 32571
9	Iguape Cateto	IRGC 4122	33	Milyang 23	IRGC 34393
10	Sintane Diofor	IRGC 5418	34	Matia Aman 53-13	IRGC 37764
11	N 22	IRGC 6264	35	Black Gora	IRGC 40275
12	Basmati 370	IRGC 6426	36	Pacholinha	IRGC 50531
13	Kalukantha	IRGC 7755	37	Bala	IRGC 50927
14	Kun Min Tsieh Hunan	IRGC 8195	38	CO 39	IRGC 51231
15	RTS 12	IRGC 8234	39	Chodongji	IRGC 55471
16	Pokkali	IRGC 8948	40	Patbyeo	IRGC 55607
17	Vary Vato 462	IRGC 10964	41	Chhote Dhan	IRGC 58930
18	Fandrapotsy 104	IRGC 10984	42	Lemont	IRGC 66756
19	Moroberekan	IRGC 12048	43	Aus Jota	IRGC 66767
20	ARC 10177	IRGC 12386	44	Jing Xi 17	IRGC 67676
21	IR36	IRGC 39292	45	Rayada	IRGC 77210
22	Yancaoussa	IRGC 16071	46	Teqing	IRGC 81093
23	Khao Kap Xang	IRGC 23423	47	IR64	IRGC 66970
24	Aswina	IRGC 26289	48	Nipponbare	IRGC 12731

Celery juice extract (CJE) was produced by the technique of Till et al. (2004). The optimal amount of CJE required per reaction was determined by titration using a known mismatch (SNP) for TPP between Nipponbare and Kun Min Tsieh Hunan. Various dilutions of CJE ranging from 0 to 5 μl CJE in CJE buffer were prepared and used for mismatch cleavage of normalized PCR products. The optimum amount of CJE for cleavage was identified as that resulting in clear bands in both channels against a faint background for either Li-Cor or agarose based detection (Fig. 1). The amount required for agarose detection was found to be about ten times that needed for detection on the Li-Cor DNA analyzer.

With the batch of CJE used for the experiments, an enzyme mixture was prepared by mixing 0.015 μl CJE in CJE buffer, using 3 μl CJE buffer and 16.98 μl sterilized nanopure water, per reaction. Enzyme digestion was carried

Table 2 Genes and primer sequences used in this study

Gene name	TIGR locus ID	Primer (F-forward; R-reverse)
MYB1	LOC_Os12g41920	F-ATAATATgggTgCCCCgAAgCAgAAAT
		R-TCCTACAgTTCTCCACTTCCCCATTCC
TPP	LOC_Os02g44230	F-ggCACACTgTCgCCTATTgTggATg
		R-gTTTACgAgCCgTgCgACCAgTTTC
ADF	LOC_Os02g44470	F-CCTCCTTgCAgggCCTgTCTgT
		R-TggCAgTgAggCATTCTCATAAAA
Oxalate oxidase	LOC_Os080g09000	F-AgTTAgCTTCCCATCAgAgAAAgA
		R-ggTTgTTTgCAAgCACACATAAT
Oxalate oxidase-like protein	LOC_Os080g09010	F-CAACgATAAACACAACCTgTTAgC
		R-ggggATACCACACTAgAACAACAT
Oxalate oxidase	LOC_Os080g09020	F-gCTTAATTgACTTgTTCTCCATCA
		R-AgTCCgTAAgCggACAATATTTAg

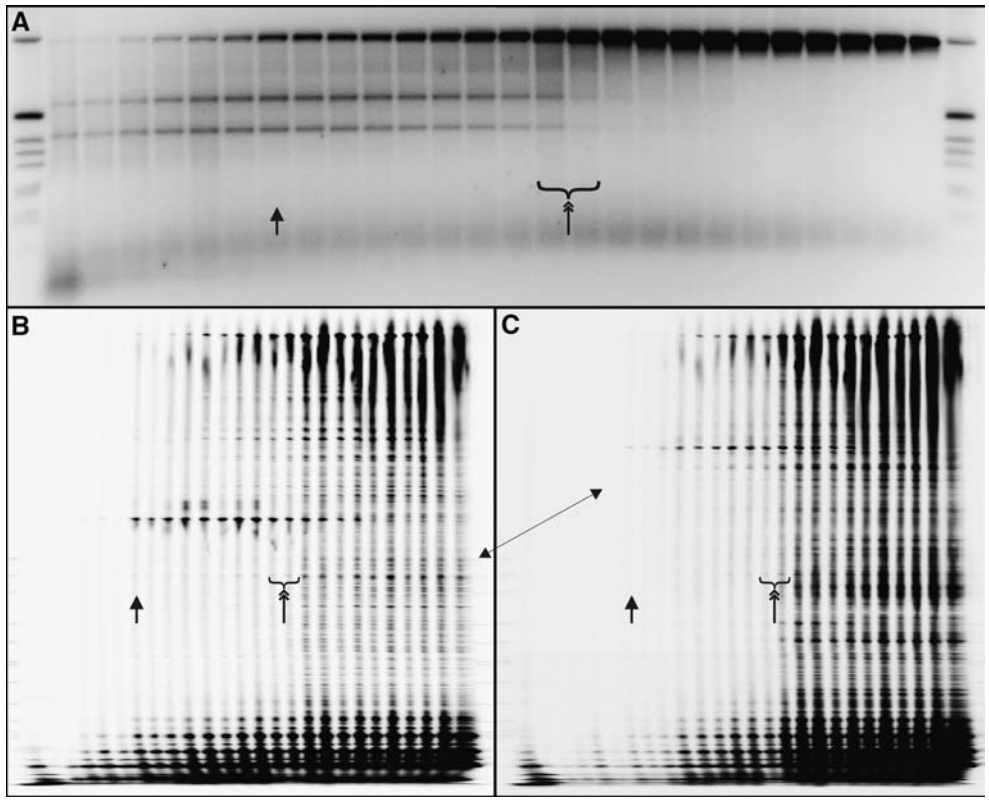

Fig. 1 Determination of the optimal amount of celery juice extract (CJE) for mismatch cleavage by titration. CJE prepared according to Till et al. (2004) was diluted in CJE buffer with the following amounts (in μl) used for digestion: 5, 1, 0.5, 0.25, 0.2, 0.15, 0.1, 0.09, 0.08, 0.07, 0.06, 0.05, 0.04, 0.03, 0.02, 0.01, 0.005, 0.004, 0.003, 0.002, 0.0005, 0.00025, 0.000125, 0.0000625, 0.00003125, or 0 μl of CJE. PCR products for the TPP locus were prepared using a 1:1 mixture of Nipponbare to Kun Min Tsieh Hunan DNAs and digested as described for agarose-based detection (**a**) or for Li-Cor detection (**b**, **c**, IRD700 and IRD800 channels, respectively). The *bold arrows* point to the lane with 0.1 μl CJE (best for agarose) and the *double-headed arrow* points to between the lanes with 0.02 and 0.01 μl CJE (best for Li-Cor). The *line drawn between panels* **b** *and* **c** indicates the paired bands of the SNP between Nipponbare and Kun Min Tsieh Hunan. The external lanes are 1 kb ladder for panel **a** or IRDye ladder for panels **b** and **c**

out at 45°C for 15 minutes. Enzyme activity was stopped by adding 5 μl of 0.225 M EDTA and mixed thoroughly. To remove excess salt and concentrate the samples, 3 μl of 3 M sodium acetate and 60 μl isopropanol were added to each reaction, mixed thoroughly, and incubated at –80°C for at least an hour. The samples were centrifuged at $5{,}180 \times g$ for 30 min and the supernatant discarded. The pellet was washed with 75% ethanol, centrifuged at $5{,}180 \times g$ for 10 min and dried at 65°C for 10 min. To each sample, 5 μl formamide loading dye and 10 μl nanopure water were added and concentrated to about 1.5 μl by evaporation at 85°C. Samples were loaded (0.3 μl/well) on a 6.5% polyacrylamide gel. The gel was run at 1,500 V for 5 h on the Li-Cor 4300 system.

During electrophoresis the Li-Cor genotyper captured two images, one each for the IR700 and IR800 channels. Cleaved products were detected by visual inspection of the images from both channels. Dark bands that appeared in both the channels but at varying positions and that have a total inferred molecular weight equivalent to that of the full length PCR product were considered indicative of a SNP. Bands in the middle of the gel (50% of the size of a full length product) were also considered indicative of a SNP, even though no apparent shift in mobility can be seen by comparing images from the two channels.

EcoTILLING on agarose gel

PCR amplification was similar to that described above except that the final volume of the reaction was 14 μl and unlabeled primers were used. The conditions for PCR amplification, denaturation, and renaturation were the same as used for EcoTILLING with the Li-Cor DNA analyzer. The CEL I digestion procedure was modified as each reaction was treated with the enzyme mixture that consisted of 0.1 μl CJE, 1.5 μl CJE buffer, and 8.3 μl nanopure water. The digestion was carried out at 45°C for 30 min. Enzyme digestion was stopped as described above and 10 μl of the digested product was resolved on a 1.4% agarose gel in 0.5× TBE (Tris–borate EDTA), run at 5 V/cm. The gel was stained with 0.5× g/ml ethidium bromide and visualized under a UV-transilluminator. Samples that exhibited bands in addition to the full-length product indicated the presence of SNPs in the pool.

Note that a larger amount of CJE is used for EcoTILLING assays performed on agarose as compared to those performed on LiCor genotypers. A partial digest would result in cleaved products tagged to the labeled primers (5′ and 3′ ends of the PCR product) whereas a complete digest would result in fragments that are not labeled and would not be read by the genotyper but are detectable by regular staining in agarose gels. Thus, for detection on the Li-Cor with denaturing PAGE, partial digests are attempted since nicked products are sufficient for detection. The wide dynamic range of the IRDyes (http://www.licor.com/bio/IRDyes/IRSeq.jsp) also allows greater sensitivity in the detection of products with lower abundance. In the case of detection on agarose, complete digestion with both strands cleaved at a mismatch position is preferred to maximize the amount of cleaved fragments.

Mapping

Because of the abundance of SNPs, EcoTILLING can be used for mapping specific genes. In a mapping experiment, a candidate gene for host defense is amplified from mixed genomic DNA of the parental lines, after which the PCR product is subjected to heteroduplex formation and digested with CEL I endonuclease. The presence of cleaved products indicates SNPs between the alleles of the parental lines. Secondly, genomic DNA of individual lines of the corresponding mapping population is combined with that of either parents and used as template for EcoTILLING of the candidate gene. The absence of cleaved products indicates that the line carries the same allele as that of the parent that it was combined with, while the presence of cleaved products indicates the alternative allele.

Two lines—Shanhuangzhan-2 (SHZ-2) and Lijiangxin-tuan-heigu (LTH)—of a recombinant inbred (RI) population for disease resistance analysis (Liu et al. 2004) were used to test this procedure. The two DNA samples were combined in a 1:1 ratio and used as a template to detect SNPs between the parental alleles for two oxalate oxidase loci (LOC_08g09000 and LOC_08g09020). DNA from the individual parental lines was also used as template to check if the parental lines were homozygous for the target loci. We first tested for SNPs between the parental lines for oxalate oxidase (LOC_08g09000 and LOC_08g09020) and then used the same loci to screen recombinant inbred lines (RILs) exhibiting extreme phenotypes. Based on phenotype data for disease reaction against the blast isolates GDV-1 (Guangdong, China) and PO6_6 (Philippines) (Liu et al. 2004), we selected eight RILs that exhibited extreme resistance and ten RILs that exhibited extreme susceptibility to the isolates. For LOC_08g09000, DNA of each RIL was combined with DNA of SHZ-2, and for LOC_08g09020, DNA of each RIL was combined with that of LTH. DNAs from individual RILs were assayed to confirm that the loci were homozygous.

Results and discussion

TILLING/EcoTILLING provides an innovative approach to survey induced or natural variation in the genomes (Comai et al. 2004; Gilchrist and Haughn 2005; Winkler et al. 2005). A main appeal of the technique is the ability to pool DNA samples such that multiple genomes can be surveyed in an efficient manner. Any attempts to

improve the technique must take into account the pooling and genome scanning efficiency and, ultimately, the cost-effectiveness of the procedure. Although the use of agarose electrophoresis is obviously a simplification of the ''conventional'' TILLING method, it is not immediately obvious that the efficiency parameters are not compromised. We therefore experimentally tested the important parameters throughout the procedure: pooling efficiency, size of scanning windows, and overall robustness of the method.

Pooling efficiency

To determine the efficiency of pooling using agarose gel detection, we conducted a reconstruction experiment using the gene for the oxalate oxidase-like protein on chromosome 8 (LOC_Os08g09010) and two mutants of IR64 that are known to have homozygous or heterozygous alleles at this locus as compared to the wild type. The gene for the oxalate oxidase-like protein was amplified from the wild type IR64, a homozygous M_3 line M715-1 or its heterozygous M_2 parent M715, and pools of the two DNA samples at ratios of 1:1; 1:3, 1:7, 1:11, 1:15, 1:19, 1:23, 1:27, 1:31, 1:35, 1:39. As the pool size increased to 1:23, the cleaved products faded. The results suggested that eightfold pooling (1:7 alleles) was optimal (Fig. 2), but cleaved products were still visible up to the pool ratio of 1:19. In this case the concentration of the PCR product of the gene for the oxalate oxidase-like protein was 50 ng/μl. Detection in eightfold pools was confirmed for the amplicon from another oxalate oxidase like protein locus, LOC_Os08g09040 (Supplementary Fig. 1). Similar results to these were obtained for pooling DNAs of IR64 and Kun Min Tsieh Hunan DNAs using both Li-Cor and agarose based detection (data not shown).

Since heterozygous mutations are most common in an M_2 population, we further tested the sensitivity of detecting a known heterozygous mutation in a pool of homozygous plants (Fig. 2). DNA from a mutant M715 which carries a heterozygous mutation (A/a) at the locus encoding oxalate oxidase-like protein was mixed with wild type DNA (AA) to reconstitute different allelic ratios. Cleaved DNA fragments were detectable in 15A:1a, suggesting that a single heterozygous mutant can be detected from a DNA pool of eight M_2 plants (Fig. 2). To demonstrate that the pooling strategy was indeed effective in practice, we made DNA from pools of eight plants using 800 EMS-induced M_2 mutants, and were able to detect heteroduplexes in 11 genes screened so far (see Supplementary Fig. 2 for detection of mutations at three gene loci).

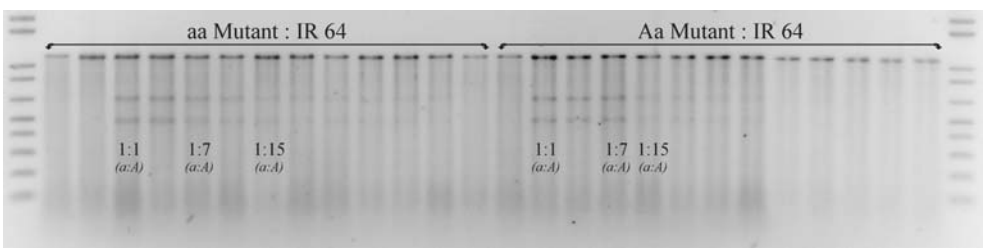

Fig. 2 Evaluation of pool capacity for detecting CEL I-cleaved products from homozygous and heterozygous alleles on agarose gel. DNA from the heterozygous M_2 mutant M715 and a M_3 homozygous selection from this mutant M715-1 were pooled to wild type IR64 at ratios of 0:1, 1:0, 1:1; 1:3, 1:7, 1:11, 1:15, 1:19, 1:23, 1:27, 1:31, 1:35, 1:39 for *Lanes 2–14* (M715-1) and for *Lanes 15–27* (M715). The locus encoding oxalate oxidase-like protein on chromosome 8 (LOC_Os08g09010) was amplified by PCR and the PCR products were subjected to heteroduplex formation followed by CEL I treatment. *Lanes 2–14* contain the CEL I-cleaved products from M715-1 while *lanes 15–27* contain those from M715. *Lanes 3 and 16* contained products from DNA of the mutants alone. No cleavage is seen in *Lane 3* while cleavage is observed in *Lane 16*, confirming that mutant M715-1 is homozygous whereas M715 is heterozygous at the oxalate oxidase-like protein locus. The ratios indicate the proportion of mutant allele (a) and wild type allele (A) in the pool. Heteroduplex DNA formed by the heterozygote is detectable in 1a:15A, suggesting that a single heterozygous mutant can be readily detected from DNA pool of 8 M_2 plants. The full length product is 1,120 bp. *Lanes 1 and 28* are 1 kb plus ladder

Our result is comparable to prior reports on TILLING mutant populations where eight DNA samples are pooled prior to PCR amplification of genomic region under query (Colbert et al. 2001; Henikoff et al. 2004). The efficiency of heteroduplex formation is directly proportional to the pool size (Yeung et al. 2005). Greber et al. (2005) were able to detect CEL I-cleaved fragments of PCR products generated from pools of clones in a 1/16 ratio on agarose gel. Our results indicate that, conservatively, mismatches can be detected in a pool of 8 mutants; however, the gel image (Fig. 2) suggests that deeper pooling (up to 1:23 or higher) may be achievable with sufficient control of DNA template concentrations, optimization of PCR amplification, and CJE cleavage conditions. Gel densitometry of the lanes in Fig. 2 detected peaks at ~5% intensity in the 1:35 mixture as compared to those in the 1:7 mixture (data not shown). Hence, it seems that the depth of pooling can be further improved with a more robust means to score gel images.

In order to work with larger pools of ten or more DNA templates, the PCR reactions need to be optimized to obtain higher yields of up to 200 ng/μl because the ratio of PCR products in a pool is directly proportional to the ratio of the templates (Ruano and Kidd 1992). If the yield of PCR product, derived from pool of eight DNA samples, was at a concentration of 100 ng/μl, then each template would contribute about 12.5 ng/μl (100/8). Theoretically, this should be sufficient for the visualization of the cleaved products in agarose gels. However, it should be noted that CEL I cleaves heteroduplexes at only about 20% efficiency (Yeung et al. 2005). Furthermore, the number of SNPs in a pool would affect production of cleaved products with a given cleavage efficiency of CEL I. When a single SNP is present in the amplicon (e.g., in mutants), a higher concentration of cleaved products is expected than when the activity of CEL I is distributed across multiple mismatch sites within an amplicon (e.g., in germplasm). Thus, with the agarose gel method, it is essential to maximize the yield of PCR product so that a sufficient amount of cleaved products can be visualized by staining.

Unlike screening mutants, EcoTILLING is applied to germplasm where it is common to observe a large number of SNPs between any two genotypes. We chose to contrast each germplasm accession separately against two reference genotypes that are distinct in their genomic sequence. For the reference genotypes, we used the *indica* var. IR64 and the *japonica* var. Nipponbare where complete genome sequence is available (International Rice Genome Sequencing Project 2005). It is likely that if no SNPs are found in a particular genotype paired against IR64 then there would be a high possibility of detecting a SNP when the genotype is contrasted with Nipponbare. However, in certain cases, SNPs are detected against both IR64 and Nipponbare indicating diverse haplotypes present in the germplasm. Given the high levels of variation in diverse germplasm, pooling more than two samples should be avoided. Furthermore, pooling multiple genotypes (depending on the diversity of the genotypes) may not be efficient considering the need to deconvolute the pooled samples. Thus, the need for pooling efficiency for screening germplasm (EcoTILLING) is less critical than that for TILLING mutants. Yet, if genotypes are closely related, the frequency of natural SNPs might approach that of chemically induced mutants, allowing for higher levels of pooling in EcoTILLING without sacrificing efficiency.

Larger scanning window

Typically for TILLING/EcoTILLING on the Li-Cor genotyper, the recommended amplicon size for scanning is between 1,200 and 1,500 bp. In the Li-Cor genotyper a laser beam scans a narrow window many times as the gel is running. When a product runs past this window the laser captures the image and these series of images are then compiled to create a gel image. Therefore, the gel is run for a long time (about 5 h) until the full length product is captured. In instances when the amplicon is 2 kb or more, the time taken to run the polyacrylamide gel would be far greater in comparison to that on an agarose gel.

We showed that it was possible to detect SNPs on agarose gels in amplicons greater than 2 kb (Fig. 3). The MYB1 gene (Table 2) was amplified individually from Nipponbare, IR64, and Azucena and also from pools of IR64 and Nipponbare

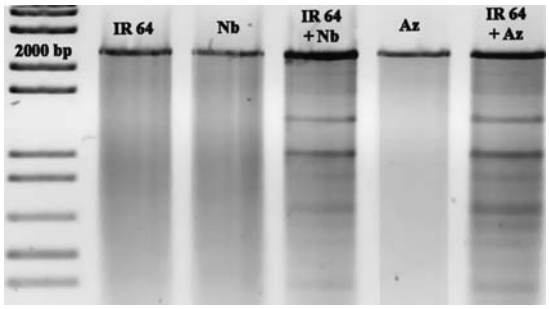

Fig. 3 TILLING of 2,369 bp amplicon on agarose. The coding sequence of MYB1 was amplified and subjected to heteroduplex formation followed by CEL I treatment. The full length product is 2.3 kb. *Lane 1* 1 kb plus ladder; DNA templates used: *lane 2* Nipponbare (Nb), *lane 3* IR64, *lane 4* IR64 and Nb (1:1), *lane 5* Azucena (Az), *lane 6* IR64 and Az (1:1)

(1:1) and IR64 and Azucena (1:1). CEL I-cleaved products were detected in both pools, indicating SNPs between IR64 and Nipponbare and also IR64 and Azucena for the MYB1 locus. For most genes, it is feasible to design primers to amplify unique fragments of 2 to 3 kb. This ability to scan larger genomic regions should improve the efficiency in surveying across gene structures. Based on our experience, when the SNPs are close to the 5′ or 3′ ends of an amplicons (i.e., within 50 bp from the ends), it is difficult to recognize the cleaved products on either the Li-Cor or agarose-based systems. Therefore, two or more sets of primers are designed to cover the entire genomic region of interest such that one amplicon overlaps the adjacent amplicon by at least 100 bases.

Making calls on Li-Cor versus agarose images

DNA samples from 48 genotypes (Table 1) contrasted to IR64 or Nipponbare were used to detect SNPs. We used the genomic region of the TPP and ADF genes to make calls across the germplasm. The calls made by using the Li-Cor genotyper for the TPP and ADF genes were identical to those made from agarose gels (Fig. 4a, b). In the case of the ADF gene, two of the cleaved products did not resolve on agarose gels while they were visualized as two distinct, but close bands in the Li-Cor images (Fig. 4b). This illustrates that haplotyping based on agarose gel band patterns would not always be feasible on standard agarose gels due to lower resolution. Thus, observing cleaved products on an agarose gel only helps to conclude that SNPs are present among the contrasted samples.

As discussed earlier, for the purpose of haplotyping, the cleaved products can be resolved on a polyacrylamide gel followed by staining with SYBR green (Yang et al. 2004). However, to confirm the number, position and type of SNPs, the PCR amplicons need to be sequenced regardless of the detection method. For most applications, it is not essential to estimate the number of SNPs in the amplicon or the position of the SNPs prior to sequencing. Agarose gels are often sufficient to test for the presence or absence of SNPs in a DNA pool. The number and position of the SNPs are thereafter identified by sequencing.

Genetic mapping

In addition to screening germplasm and mutants, one potential application of the simple SNP-detection technique is to overcome the problem of low polymorphism in specific target genes in the analysis of genetically related materials. We scanned a 2,369 bp region of the MYB1 gene from two *indica* parental lines SHZ-2 and Texianzhan-13 (TXZ-13) used in disease resistance breeding (Liu et al. 2004). Figure 5 shows the detection of SNPs between the two parental lines for the MYB1 locus even though these two lines are from genetically similar pedigrees. We also showed the presence of SNPs in the MYB1 locus between Azucena (*japonica*) and SHZ-2. We used the MYB1 locus to assay the allele carried by a BC_3F_3 line (BC10) by contrasting BC10 to each of the parental lines (SHZ-2 or TXZ-13). Our results indicated that BC10 carried the TXZ-13 type allele (Fig. 5) suggesting that the technique could be applied in backcross programs to select for the target allele.

To validate this technique for mapping, we tested the segregation of two loci—oxalate oxidase LOC_Os08g09000 and LOC_Os08g09020 (members of the same gene family)—that were previously shown to be associated with disease resistance in a RI population derived from SHZ-2 and LTH (Liu et al. 2004). The presence of SNPs between the parental alleles for the two loci was

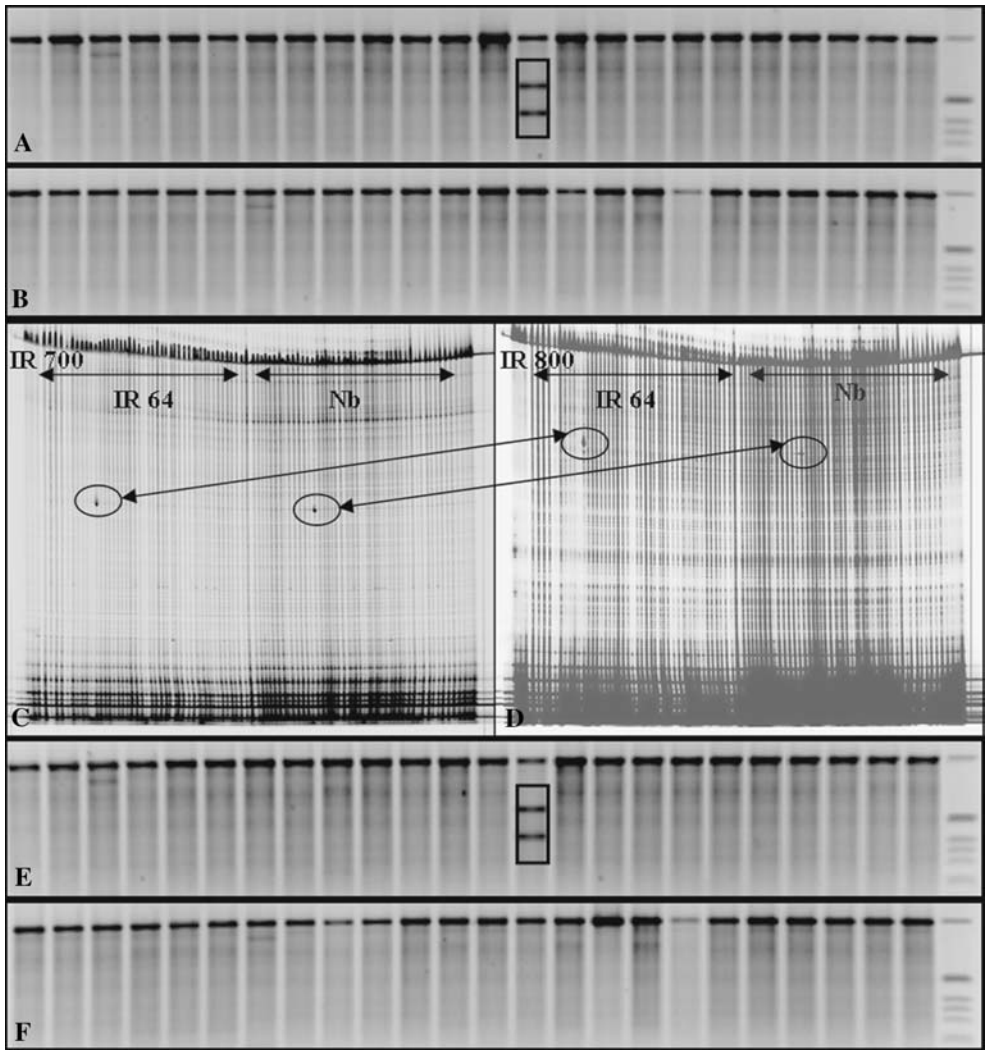

Fig. 4 **a** EcoTILLING of the TPP gene on Li-Cor and agarose. The figure shows a perfect match of SNP detection calls between the two platforms for the TPP gene. **a**, **b**, **e**, **f** Agarose gel pictures of CEL I-cleaved products. The 48 varieties (Table 1) were contrasted with IR64 (**a**, **b**) or Nipponbare (Nb) (**e**, **f**) and subjected to CEL I treatment. **a** Represents the first 24 varieties and **b** represents varieties 25–48 (*left to right*) contrasted with IR64. **e** Represents the first 24 varieties and **b** represents varieties 25–48 (*left to right*) contrasted with Nb. The *boxes* indicate SNPs between IR64 or Nb and Kun Min Tsieh Hunan (IRGC accession 8195). **c**, **d** Li-Cor images from the IR700 and IR800 channels, respectively. *Lanes 1–48* represent the 48 varieties contrasted with IR64 while *lanes 50–98* represent those contrasted with Nipponbare (Nb). CEL I-cleaved products indicating SNPs between IR64 or Nipponbare and Kun Min Tsieh Hunan are *circled*. *Double headed arrows* point to the presence of complementary bands in both of the channels (**b**). EcoTILLING of the ADF gene on Li-Cor and agarose. The same germplasm varieties and contrasts were performed as those in Fig. 5a, see legend for details. CEL I-cleaved products indicating SNPs were identified between IR64 or Nipponbare and Chondongji (IRGC accession 55471). *Single-head arrows* indicates doublets (differing by a few bases) seen in the Li-Cor IR700 and IR800 channels that are not resolved in agarose. The *asterisk* indicates a band at about 50% full length. No mobility shift is seen between the channels, this band might have been considered artifactual by the ''squint'' procedure

identified by pairing alternative alleles in a TILLING assay (Fig. 6). Using this assay on the RI progeny, we confirmed genetic linkage of the loci with disease resistance. All eight resistant RI lines carried the SHZ-2 allele while eight of the ten susceptible lines carried the LTH allele for

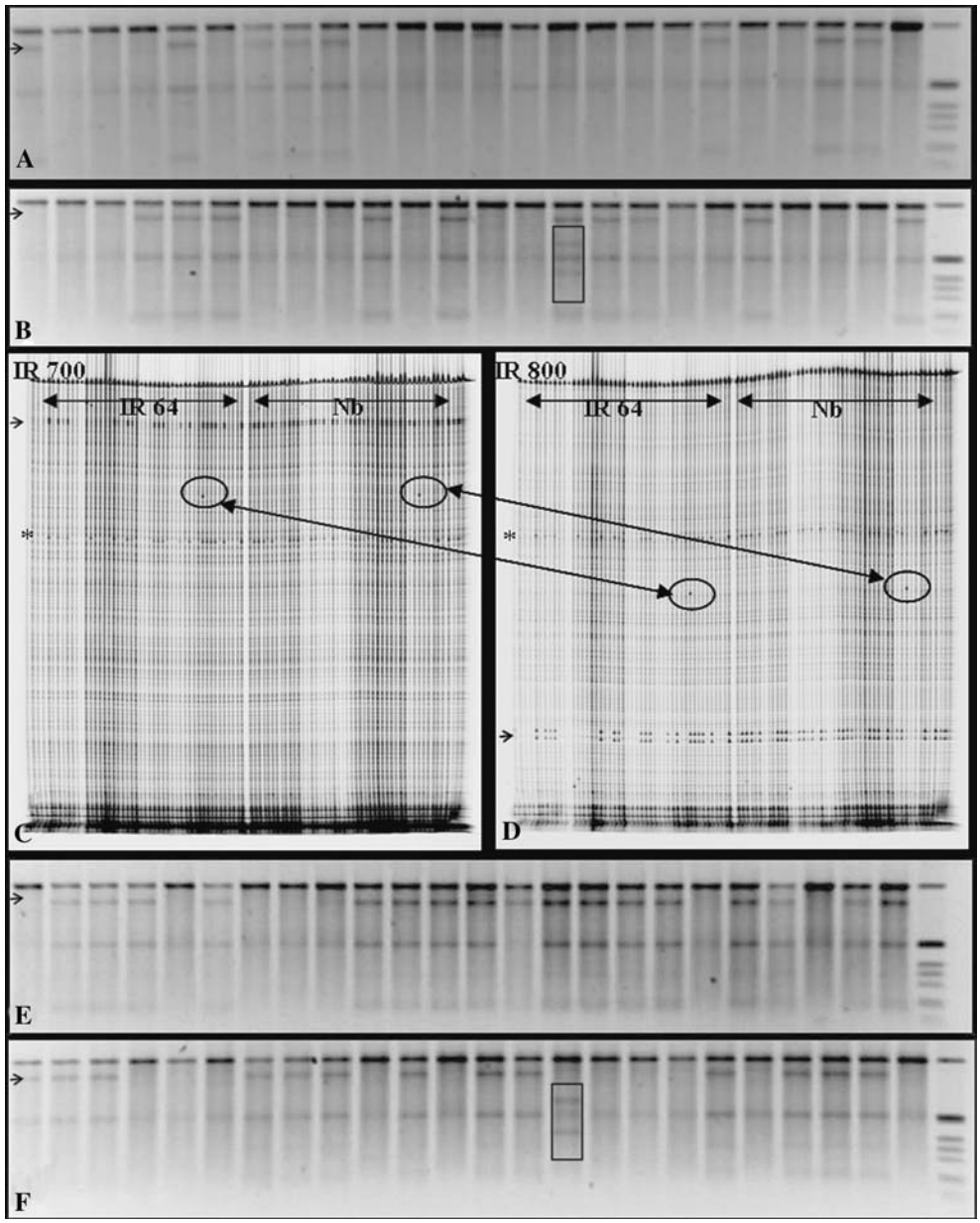

Fig. 4 continued

both loci. One of the remaining susceptible RI lines carried the SHZ-2 alleles and the other line was heterozygous at both loci (Fig. 6).

Given the clear association between the parental alleles and the extreme phenotypes, we analyzed the entire RI population of 215 lines. Single marker analysis was performed using the SNP marker and trait data (Table 3). The R^2 values were consistent with the previous QTL mapping results (Liu et al. 2004). Although the two loci are separated only by about 10.5 kbp, a small difference in R^2 values associated with each locus was observed. This can be attributed to the greater ability to assay polymorphism and hence, detection of recombination within the region.

Together, these results suggest that mismatch detection offers a simple way to track specific genes in breeding pedigrees and segregating

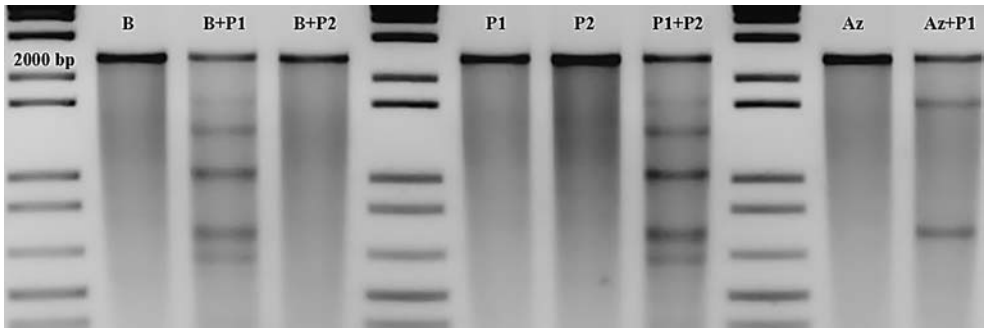

Fig. 5 SNP detection among Sanhuangzhan 2 (SHZ-2) (*indica*) (P1); Texianzhan-13 (TXZ-13) (*indica*) (P2), BC_3F_3 line (parental lines SHZ-2 and (TXZ-13) BC10 (B) and Azucena (Az) for the MYB1 gene. *Lane 1, 5 and 9* 1 kb plus ladder; DNA templates used: *lane 2* BC10; *lane 3* BC10 and SHZ (1:1); *lane 4* BC10 and TXZ-13 (1:1), *lane 6* SHZ-2, *lane 7* TXZ-13, *lane 8* SHZ-2 and TXZ-13 (1;1); *lane 10* Az; *lane 11* Az and SHZ-2 (1:1). The PCR products were subjected to heteroduplex formation followed by CEL I cleavage. SNPs were detected between the BC10 and SHZ2 (indicating the allele carried by BC10 for MYB1 was of TXZ-13 type); SHZ-2 and TXZ-13; and also SHZ-2 and Az as evidenced by the cleaved products. The full length product is 2,300 bp

populations without an upfront investment in sequencing. However, it should be noted that the method may be prone to the problem of misclassifying alleles due to residual heterozygosity in a RIL. Also, artifactual bands could be interpreted as a cleaved heteroduplex. To deal with these problems, individual lines of the mapping population should be checked independently to identify any residual heterozygosity. Second, DNA of the RIL can be paired reciprocally with both parents to provide complementary data on the allelic state.

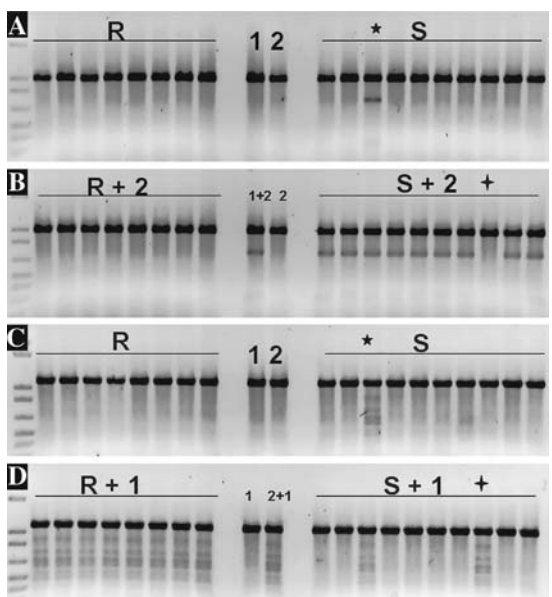

Fig. 6 Detection of parental allele for Os0809000 (**a**, **b**) and Os009020 (**c**, **d**) carried by the 18 recombinant inbred (RI) lines that exhibited extreme phenotypes. *R* resistant; *S* Susceptible; *1* LTH; *2* SHZ-2. A *star* indicates a susceptible RIL that is heterozygous for both loci, and a *cross* indicates a putative RI lines. **a**, **c** Individual lines; **b** the DNA of each line was combined with DNA of SHZ-2; **d** the DNA of each line was combined with DNA of TXZ-13

EcoTILLING has been recognized to have potential application in increasing the power of QTL and association mapping, but its application so far has been limited due to its relatively high cost and accessibility of genotyping infrastructure (European Plant Science Organization 2005). The simplified agarose method would prove useful in cases where parental lines are closely related and polymorphic molecular markers are difficult to find. The approach can be used for analyzing target genes or genomic regions in NILs (near-isogenic lines), BILs (backcross inbred lines), or in MAB (marker-assisted back-crossing) produced lines.

PCR quality and quantity

To take advantage of agarose gel detection, special attention must be given to the quality and quantity of PCR products. First, it is important that a single specific product is obtained from a PCR reaction since the presence of non-specific

Table 3 Single-marker analysis for candidate genes encoding members of the oxalate oxidase family

Locus		Disease phenotype[a]			
		Lesion density	Lesion size	Diseased leaf area	
				IRRI glasshouse	Guangdong glasshouse
Os08g09000	R^2(%)	5.7	6.7	21.3	19.6
	P-value	0.007	0.004	<0.001	<0.001
Os08g09020	R^2(%)	4.5	6.7	19.1	25.4
	P-value	0.016	0.004	<0.001	<0.001

[a] Phenotype data from Liu et al. (2004)

products may lead to heteroduplex formation. Non-specific amplification would result in the detection of CEL I-cleaved products even in the control samples. Therefore, it is important that once a SNP is detected in a pool, the individuals of the pools be tested separately to confirm that the cleaved products are a result of heteroduplexes formed across members of the pool and not a result of non-specific amplification.

Secondly, as mentioned in the pooling section, it is critical to have a high yield of the amplicon. Unlike that of the Li-Cor genotyper, the sensitivity of detection on agarose gels is low. Products at concentrations less than 5 ng/μl would not be detected on regular agarose gels. A comparison of these and other features of the two systems is shown in Table 4.

Conclusion

We have evaluated a simplified procedure to detect CEL I-cleaved products and extended its application to genetic mapping studies. The method is robust and does not require sophisticated instrumentation or costly labeled primers. This will enable more laboratories, particularly in the developing countries, to apply the SNP detection technique method in their work and thereby accelerate the discovery of gene function and candidate gene mapping.

We expect the technique to have broad applications as genome sequence information continues to expand for multiple plant species. Many more candidate genes are expected to be identified from genome-wide expression studies or from comparative analysis across species. However, the paucity of polymorphism often prevents the use of these genes in association or mapping analyses. Provided that enough sequence information is available to obtain PCR-amplicons, mismatch detection offers a simple way to track specific genes in breeding pedigrees and segregating populations without upfront investment in sequencing. The ease in detecting SNPs in related genetic materials implies that many more candidate genes can now be used for mapping and pedigree analyses. Because conserved orthologous markers are often low in variation, the use of SNP detection may provide a useful bridge to evaluate orthologous markers across species.

Table 4 A comparison of properties of the agarose-based system to the Li-Cor analyzer

Parameter	SNP detection on agarose gel	SNP detection using the Li-Cor genotyper
DNA pooling	2 per pool for EcoTILLING; 8 per pool for TILLING	2 per pool for EcoTILLING; 8 per pool for TILLING
PCR primers	Unlabeled	Labeled (IRD 700 and IRD 800)
Amplicon size	2–3 kb	1.2–1.5 kb
Post-CEL I cleavage clean up	None	Removal of excess salt and sample concentration
Gel analysis	Agarose/ethidium bromide	Polyacrylamide gel/Li-Cor genotyper
Overall time for electrophoresis	2 h	5 h
Resolution of closely spaced SNPs	May not be distinguishable	Distinguishable
Relative cost	Low	High

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