TILLING and Ecotilling for Rice

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Abstract

Mutagenesis is frequently used to test gene function and to aid in crop improvement. Targeting Induced Local Lesions in Genomes (TILLING) is a reverse genetic strategy first developed to identify induced point mutations in *Arabidopsis*. This general strategy has since been applied to many plant and animal species. Here, we describe a protocol for high-throughput TILLING in rice. Gene segments are amplified using fluorescently tagged primers, and products are denatured and reannealed to form heteroduplexes between the mutated and wild-type sequences. These heteroduplexes are substrates for cleavage by single-strand-specific nucleases. Following cleavage, products are analyzed on denaturing polyacrylamide gels using the LI-COR DNA analyzer system. Several rice TILLING populations have been described, and a public mutation screening service is now available. The basic methods used for TILLING can be adapted for the discovery and cataloguing of natural nucleotide variation in populations, a strategy known as Ecotilling, which was first used to study genetic diversity among *Arabidopsis* ecotypes, and has since been applied to crop plants.

Key words: Enzymatic mismatch cleavage, Mutation discovery, Reverse genetics, Nucleotide polymorphism, Celery juice extract

1. Introduction

Full genomic sequences are now available for many organisms; however, the task of assigning function to each gene remains a sizeable effort for many species. Sequence homologies and transcriptional profiles provide important clues to gene function, but proof of *in vivo* activities requires further work. A powerful strategy for assigning *in vivo* gene function is the phenotypic characterization of gene disruptions caused by induced and natural nucleotide variation. Indeed, this has been a major source of our understanding of fundamental biological principles, and has provided a means for crop domestication and breeding (1).

Reverse genetic methods utilize knowledge of gene function and accumulation of genome sequences to target specific genes for

disruption without the *a priori* need of phenotypic characterization (2). Large mutant populations are stored in early generations and only desired mutants are chosen for phenotypic characterization and genetic analysis. Additionally, reverse genetic strategies allow for the identification of alleles that confer no phenotypic consequences on their own, but do when combined with other alleles. This provides a mechanism for the development of traits that are either unlikely or impossible with traditional forward methods.

Targeting Induced Local Lesions in Genomes (TILLING) is a reverse genetic method that allows the rapid screening of thousands of mutagenized lines for mutations in a gene of interest (3, 4). In TILLING, DNA from mutagenized lines is pooled and arrayed in microtiter plates (Fig. 1). A target of ~1.5 kb in a gene of interest is amplified from the pooled DNA using fluorescently labeled PCR primers. The PCR products are denatured and reannealed so that heteroduplexes can form if a mutation is present. Heteroduplexes are cleaved using a crude protein extract from celery containing the nuclease CEL I, and the products are electrophoresed on denaturing polyacrylamide gels and visualized using fluorescence imaging. Mutations are identified by the presence of cleaved PCR products in a given pool. This enzymatic mismatch cleavage method is suitable for the recovery of single-nucleotide mutations and small indels, and has been used for the discovery of thousands of induced mutations (5, 6).

The success of TILLING is dependent on two factors: mutagenesis and mutation discovery. Mutagenesis can be performed with any mutagen that yields single-nucleotide changes or small indels. Chemical mutagens have been widely used to develop mutagenized TILLING populations in a variety of species, and much has been learned with regard to the spectrum and density of mutations from different chemicals (6). Ethylmethanesulfonate (EMS) and methylnitrosourea (MNU) have both been successfully used as mutagens in rice, and populations with densities between one mutation per 135 kb and one per 290 kb have been reported (7, 8). Success with rice has led to the development of a public TILLING service (http://tilling.ucdavis.edu/index.php/Rice_Tilling). Less is known about the applicability of ionizing radiation for reverse genetics using enzymatic mismatch cleavage. Gamma irradiation

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Fig. 1. (continued) twice on the 96-well plate, but in two unique pools. The desired target is PCR amplified from the pooled DNA with two labeled primers, one label for each LI-COR channel (filled star = IRDye 700, open star = IRDye 800). The PCR products are heated to denature the DNA, and then slowly cooled to allow the formation of heteroduplexes if a mutation is present in the pool. The PCR products are then digested with Celery Juice Extract (CJE) to cut 3′ of the heteroduplex. Following sample cleanup, the products are denatured and electrophoresed on polyacrylamide gels in a LI-COR DNA analyzer. Putative mutations are visualized as above background bands (*boxes in gel image*) in the 700 and 800 channels that add up to the size of the full-length PCR product. With 2-dimensional pooling, a putative mutation will be observed twice on the same gel and will provide the identity of the individual line harboring the putative mutation. This figure is modified from ref. (7).
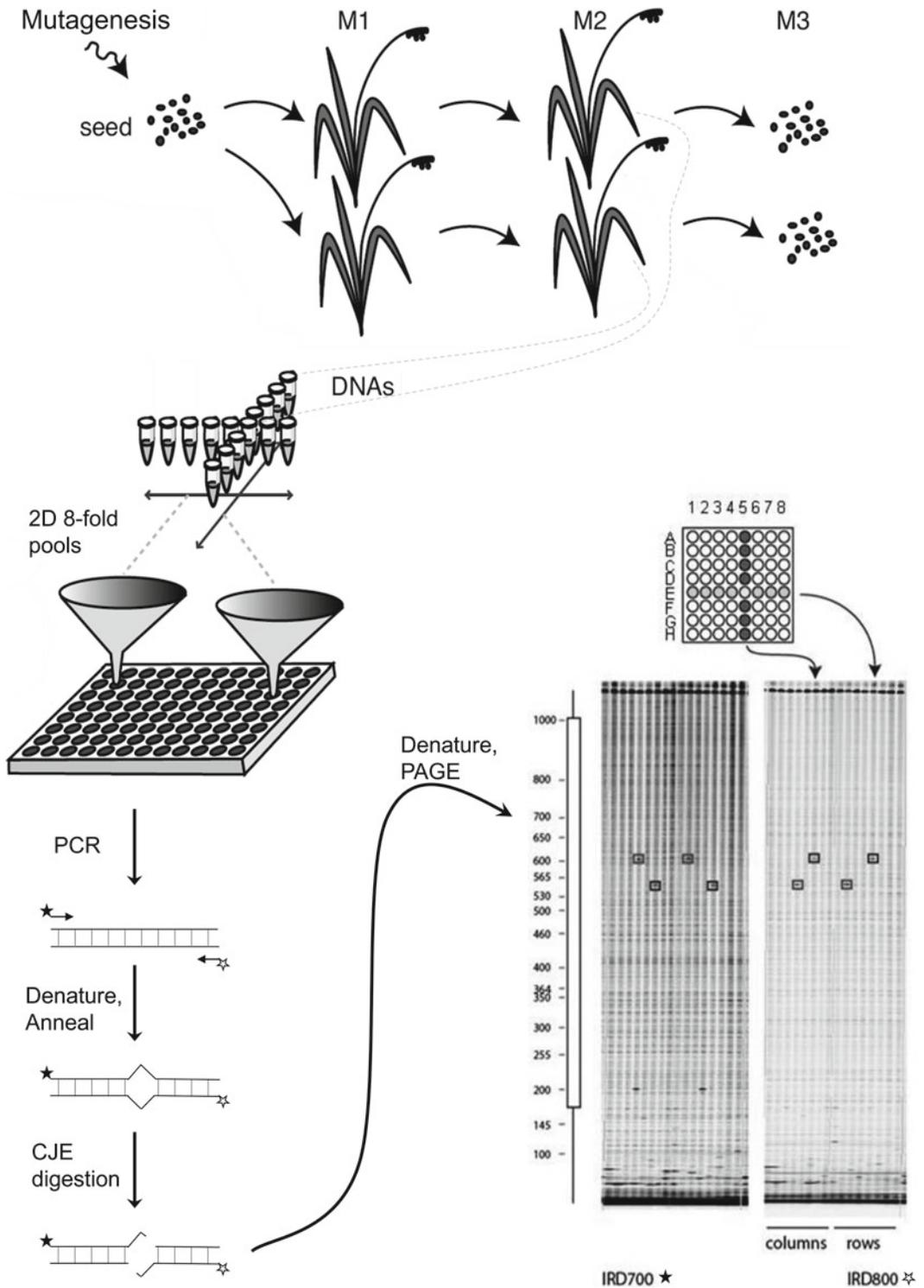
Fig. 1. Rice TILLING overview. Seeds are mutagenized and grown into M_1 plants, which are allowed to self-fertilize. Tissue is harvested from the M_2 plants for DNA isolation. The M_2 self-fertilizes and the resulting M_3 seed is saved for mutant analysis. DNA from eight M_2 plants is pooled into a well on a 96-well plate. In 2-dimensional pooling, each DNA is represented

has been used to mutagenize rice and a low density of SNP and indel mutations was reported (9). Fast neutron mutagenesis and other treatments that cause primarily large deletions can be considered useful for reverse genetics, but alternative mutation discovery methods such as PCR amplicon length polymorphism screening or array comparative genome hybridization must be used for deletion discovery (10–13). Because a high frequency of deletions may be deleterious to gene function, such mutagens also require larger populations to ensure a high probability of recovering mutations in every gene. Since mutagens causing point mutations can be used to achieve a high density of mutations randomly throughout the genome, they offer a major advantage because efficient recovery of alleles will be obtained with relatively small populations. For example, the *Arabidopsis* TILLING project averaged the delivery of ~14 mutations for every 1–1.5 kb gene target when screening between 3,000 and 6,000 mutant lines. This high throughput allows for a rapid and low-cost screening service (http://tilling.fhcrc.org/arab/status.html). Another major advantage is that point mutations can produce allelic series including knockout and missense. Hence, TILLING provides a rich source for phenotypic diversity and allows for the characterization of essential genes where knockouts would be lethal.

Several TILLING methods have been published for the discovery of point mutants. We describe here a modification for rice of the previously described method using fluorescently labeled PCR primers and a LI-COR DNA analyzer (14, 15). This method has been used since 2001 and resulted in the discovery of nearly 10,000 induced mutations by public TILLING services (6). These experiences have been used to estimate screening costs and sample throughput (16). For example, using one LI-COR DNA analyzer, ~2,300 genomic DNA samples pooled eightfold can be screened for mutations in one gene target per day. Assays are highly scalable, and with eight LI-COR analyzers, semi-automated 384 liquid handling and two dedicated technicians, 12,000 samples can be screened per day.

Further advantages of enzymatic mismatch cleavage followed by fluorescence detection using PAGE gels include simple visual detection of natural nucleotide polymorphisms that might be erroneously scored as true mutations using other methods. Additionally, multiple nucleotide polymorphisms can be discovered on a single amplicon because enzymatic cleavage does not proceed to completion. Thus, the same methods used for TILLING can be used for the discovery and characterization of natural nucleotide polymorphisms, a strategy called Ecotilling because it was first used to assay ecotypes of *Arabidopsis* (5). The method has been used for SNP discovery in organisms including rice, maize, switchgrass, humans, poplar, and melon (16–20). Major differences between TILLING and Ecotilling lie in the design of pooling and sample arraying and in data analysis as described in this chapter.

2. Materials

2.1. PCR

1. Ex-*Taq* DNA polymerase, Hot Start Version (Takara). Store at –20°C (see Note 1).

2. 10× Ex-*Taq* PCR buffer (Takara) supplied with Ex-*Taq*. Store at –20°C.

3. 2.5 mM (each) dNTPs (Takara) supplied with Ex-*Taq*. Store at –20°C.

4. 25 mM $MgCl_2$.

5. TE: 10 mM Tris–HCl, 1 mM ethylene diamine tetraacetic acid (EDTA), pH 7.4.

6. Left primer (T_m 67–73°C) unlabeled (MWG) 100 µM in TE. Store at –80°C (see Note 2).

7. Left primer (melting temperature (T_m) 67–73°C) labeled 5' with IRDye 700 (MWG) 100 µM in TE. Store at –80°C (see Note 3).

8. Right primer (T_m 67–73°C) unlabeled (MWG) 100 µM in TE. Store at –80°C.

9. Right primer (T_m 67–73°C) labeled 5′ with IRDye 800 (MWG) 100 µM in TE. Store at –80°C.

10. Primer mixture: 3 µL IRDye 700 left primer, 2 µL unlabeled right primer, 4 µL IRDye 800 right primer, 1 µL unlabeled right primer (see Note 4).

11. PCR mixture for a 96-well plate: 360 µL water, 57 µL 10x Ex-*Taq* buffer, 68 µL, 25 mM $MgCl_2$, 92 µL 2.5 mM dNTP mixture, 4 µL primer mixture, 6 µL Ex-*Taq*. Mix, adding polymerase last. Use immediately and discard the remainder after use (see Note 5).

2.2. CJE Digestion

1. Crude celery juice extract (CJE) prepared as previously described (14, 21) (see Note 6).

2. 10× CJE buffer: 5 mL 1 M $MgSO_4$, 5 mL 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5, 2.5 mL 2 M KCl, 100 µL 10% Triton® X-100, 5 µL 20 mg/mL bovine serum albumin, 37.5 mL water. Store aliquots at –20°C.

3. CJE reaction mixture for a 96-well plate: 2.4 mL water, 420 µL 10× CJE buffer, 36 µL CJE. The amount of CJE may vary based on the prep. Mix on ice, use immediately, and discard the remainder after use.

4. Stop solution: 0.225 M EDTA, pH 8.0.

2.3. Spin Plate Cleanup

1. 96-Well membrane plates (Millipore).

2. Sephadex® G50 medium (Amersham Pharmacia Biotech).

3. Sephadex spin plates: Use a Sephadex loading device (MultiScreen 45-µL column loader; Millipore; cat. no. MACL 096 45) to fill all wells of a 96-well membrane plate with approx 0.03 g of G50. Hydrate with 300 µL water. Allow the plate to stand for 1 h at room temperature. Plates may be stored at 4°C in a sealed container to prevent evaporation for up to 1 week.

4. Deionized formamide (Sigma).

5. Formamide load buffer: 38.5 mL deionized formamide, 1.5 mL 0.5 M EDTA, pH 8.0, 2.3 mg bromophenol blue. Store 1.8 mL aliquots at –20°C.

6. 200-bp markers: Perform PCR (Subheading 3.1) and spin plate cleanup (Subheading 3.3) with primers designed to yield a 200-bp fragment.

2.4. Electrophoresis

1. Ammonium persulfate (APS): Dissolve APS 10% (w/v) in water. Store at 4°C in small aliquots.

2. Polyacrylamide gel: Combine 20 mL gel mixture (6.5% acrylamide, 7 M urea; LI-COR), 15 µL N,N,N',N'-tetramethyl ethylenediamine (TEMED), and 150 µL APS. Use immediately.

3. 0.8× TBE buffer: Dissolve 89.2 g Tris-base and 45.8 g boric acid in water, add 68 mL 0.25 M EDTA, pH 8.0, bring vol to 10 L.

4. IR^2 gel analyzer, 25-cm glass plates, and 25-mm spacers (LI-COR).

5. 100-tooth membrane combs (The Gel Company).

6. Gel loading tray (The Gel Company).

7. Size Standard IRDye 700 and IRDye 800 molecular weight markers (LI-COR), or create molecular weight standards by combining PCR amplicons of desired sizes prepared as described (Subheading 2.3, item 6).

3. Methods

3.1. PCR and Heteroduplex Formation

PCR amplification should be performed using equipment and consumables that are segregated from PCR products to avoid contamination that may result in a failure to detect novel nucleotide polymorphisms.

1. Deposit 5 µL of pooled genomic DNA per well in a 96-well microtiter plate. The final concentration of genomic DNA and the maximal allowable pooling may vary depending on the organism and DNA extraction method used (see Notes 7–9).

2. Add 5 µL freshly made PCR mixture to each well. Centrifuge for 2 min at $1,400 \times g$ and place in thermal cycler.

3. Run the following thermal cycler program: 95°C for 2 min; loop 1 for 8 cycles (94°C for 20 s, 73°C for 30 s, reduce temperature 1°C per cycle, 72°C for 1 min); loop 2 for 45 cycles (94°C for 20 s, 65°C for 30 s, 72°C for 1 min); 72°C for 5 min; 99°C for 10 min; loop 3 for 70 cycles (70°C for 20 s, reduce temperature 0.3°C per cycle); hold at 8°C. After thermal cycling store samples in the dark at –20°C for use within 1 week. Extended storage may result in the loss of fluorescent signal.

3.2. CJE Digestion

1. Place PCR samples on ice and add 20 µL CJE reaction mixture to each sample. Centrifuge for 2 min at $1,000 \times g$. Mixing is not required but suggested when using 96 or 384 channel semi-automated pipettors.

2. Incubate at 45°C for 15 min.

3. Place samples on ice and stop reaction by adding 5 µL 0.225 M EDTA. Store samples in the dark at 4°C for use within 2 days or freeze at –20 C for up to 1 week.

3.3. Sample Purification

1. Assemble hydrated spin plate and an empty 96-well catch plate for centrifugation (see Note 10).

2. Spin hydrated plate containing Sephadex G50 for 2 min at $440 \times g$.

3. Remove the catch plate and insert a sample receptacle plate containing 1.5 µL formamide load dye in each well. Load the CJE digestion products onto the Sephadex plate within 10 min (see Note 11).

4. Spin for 2 min at $440 \times g$.

5. Add 200 base pair marker into sample wells representing every eighth gel lane starting with lane 4, 12, 20, and so on prior to reducing the volume. This marker facilitates lane identification and is compatible with the GelBuddy gel analysis software (Subheading 3.7, step 2). How the wells of the 96-well assay plate correspond to specific gel lanes depends on the method of sample loading (see Note 12).

6. In a fume hood, reduce the volume at 90°C to approx 1.5 µL (this takes approx 45 min), leaving formamide–bromophenol blue solution ready for loading. Transfer to ice until ready to load. Samples can be stored in the dark at –20°C for up to 1 week prior to use.

3.4. Denaturing Polyacrylamide Gel Preparation

1. Assemble 25-cm glass plates, 25-mm spacers, and casting rails. Plates can be preassembled and stored in a dust-free environment for weeks in advance.

2. Pour gels. For each 25-cm plate assembly, fill a 20-mL syringe with freshly prepared acrylamide mixture, and then dispense along the top avoiding bubbles by rapping continuously on

the plate just above the liquid edge. If any bubbles appear, remove them quickly after the gel is poured with a thin wire tool.

3. Leaving a little excess acrylamide at the well, insert the top spacer all the way into the glass, making sure that spacer is centered horizontally.

4. Insert the Plexiglass pressure plate between the glass plate and casting rails. Tighten the top screws as soon as the spacer is inserted, slightly compressing the rubber pads on the pressure plate.

5. Add acrylamide to the top glass edge where the comb is inserted and on the edges to assure that polymerization is not inhibited within the gel.

6. Let the gel set at least 90 min before placing in gel box. Gels can be stored in plastic wrap at 4°C for up to 24 h prior to use (see Note 13).

7. Prior to placing the gel in the gel box, wash the plates with distilled water, removing the comb spacer and excess polyacrylamide at the top edge. Dry the plates and wipe with isopropanol, making sure that the back plate is spotless where the laser shines through.

8. Insert the top buffer reservoir between the glass plate and the casting rails. If this is difficult, moisten the gasket with buffer and remove one casting rail in order to fit the top reservoir. Fill the lower buffer reservoir to the fill line with 0.8xTBE (approx 500 mL) and insert the gel.

9. Tighten the screws to seal the upper reservoir and fill with buffer. Rinse the slot vigorously using a large syringe without a needle (see Note 14).

3.5. Loading Samples onto Membrane Combs

1. Load 0.25 μL of sample onto the gel loading tray such that position A1 on the plate represents gel lane 3, position B1 equals lane 4, A2 equals 9, etc. Sample order will vary depending on the method of deposition, and a specific order is desirable when using eightfold pooling and two-dimensional arraying with the GelBuddy analysis program (see Note 15).

2. When the tray is filled, insert a membrane comb. Samples will absorb onto comb teeth.

3. Use a pipettor to add 0.25–0.5 μL IRDye 700 marker to comb tooth 1 and 2, and IRDye 800 marker to comb 99 and 100. This provides a means to confirm that the comb was properly inserted into the gel with tooth #1 on the left side of the gel.

3.6. Electrophoresis

1. Access the user controls (LI-COR) using an Internet browser.

2. Provide a unique gel run name, and hit Create Run (see Notes 16 and 17).

3. Start the prerun (20 min), waiting for the all ready signal from the scanner before proceeding. The prerun can be started while samples are being applied to the comb.

4. After the prerun, clean the slot out with a syringe and drain the top buffer reservoir until the level is below the glass edge. Wick out the remaining buffer between the glass plates, first with a paper towel and then with a 6-in-wide strip of Whatman 1 paper, sliding it into the slot left by the spacer.

5. Using a 1-mL pipettor, fill the slot with 1% Ficoll® leaving just a thin bead, approx 1 mm above the slot.

6. Hold the comb at a 45° vertical angle with lane 1 on the left, aim for the slot, and insert rapidly by pushing gently (see Note 18). Push the comb down until it just touches the gel surface.

7. Gently fill the reservoir to the fill line, insert the electrode cover, close the top, and then click on Collect image. The time the comb touches the slot until the time the current is applied should be no more than about 20 s or so to prevent diffusion.

8. After 10 min, open the lid (be sure that you hear the "pling" signal and the high-voltage light turns off), remove the comb, and gently rinse the slot with buffer (see Note 19). Replace the top electrode and close the lid. You should hear the pling and see the laser and high-voltage lights turn on. The gel can be monitored from a browser.

3.7. Gel Image Analysis for TILLING

1. Download GelBuddy from http://www.gelbuddy.org (see Note 20). Install and open the GelBuddy program. Under "File," select "Open 700 and 800 Channel Images." Select the corresponding 700 and 800 TIFF images (downloaded from the LI-COR). The two images can be alternated by clicking the 700/800 button.

2. Use the sliding bars at the top right to adjust the 700 and 800 images so that bands can be seen. Click the "Find Lanes" button (box with blue and red vertical lines). In the pop-up window select 96 lanes, segmented tracks, and both channels for finding lanes. Click "OK." 96 colored lines will appear over the gel lanes. Lane 1 is the first sample lane and lane 96 is the last sample lane. Blue lines indicate the lane containing the 200 bp marker (every 8 lanes starting with lane 4). Lane position can be corrected by clicking the "Lane Editing" button (box with blue and red vertical lines with arrow). Lanes can be dragged into position using the mouse. Lanes can be added or deleted by using functions in the "Edit" menu. Mark failed lanes by selecting the lane and choosing "Mark Current Lane as Failed" in the edit drop-down menu. Click the "Find Lanes" button again to eliminate the lane lines. The assigned lane number will remain.

3. Calibrate the fragment length markers (click the box with blue and green horizontal lines). Using the mouse, drag the red 100% line to the bottom of the gel. Drag the red 0% line to the full-length PCR product. Drag the blue 200 bp line to the position of the 200 bp lane marker, and the blue 700 bp line to the 700 bp marker position. Click the "Calibrate" button again to remove the calibration lines.

4. Select the appropriate mode of analysis. Click the "Find Mutations" button (box with rectangle and eye inside). In the pop-up window, fill in the full-length product size, pool size, and grouping information. Selecting "TILLING 2D" results in default settings for the grouping of row and column pool mutations.

5. Using the mouse, drag the rectangle over a putative mutation. Click the mouse to mark the mutation. Change to the other image channel and mark the corresponding band in the other wavelength image. Continue until all putative mutations are marked.

6. Under the "Report" menu, select "View Concise Report." This provides a text report summarizing the putative mutations sorted by lane or by molecular weight, as well as which lanes have equivalent molecular weight mutations. This report can be saved for future reference. More advanced features such as exporting data directly to a database are described on the GelBuddy Web site.

7. Under the "File" menu, select "Save Gel Markup" to save the marked-up gel image. This saved image can be opened by selecting "Open Gel Markup" under the "File" menu.

3.8. Gel Image Analysis for Ecotilling

1. Follow steps 1–3 of Subheading 3.7 (see Note 21).

2. Click on the "Find Mutations" button (box with rectangle and eye inside). In the pop-up window, fill in the full-length product size, pool size, and select "Ecotilling/Generic" in the presets section.

3. Using the mouse, drag the rectangle over a putative mutation. Click the mouse to mark the mutation. Change to the other image channel and mark the corresponding band in the other wavelength image. Continue until all putative mutations of the same molecular weight are marked.

4. Select the "Edit Signals Group" mode and check that all bands of the same molecular weight are grouped together. If not, alter the placement of marked bands in "Record Signals" mode. Alternatively, select all bands to be grouped in the "Edit Signals Group" mode by clicking boxed bands while holding the shift key. When all bands are selected, choose "Group Selected Signals" under the edit drop-down menu.

5. Under the report drop-down menu select "View Concise Report" and evaluate the output. Lanes grouped according to banding pattern (haplotype) are listed in the "Lane Classes" section of the report (see Note 22).

6. When complete, save the concise report and the gel markup as described in Subheading 3.7.

4. Notes

1. Alternative DNA polymerases can be considered for PCR. Hot start enzymes are advantageous because assays can be prepared at room temperature and artifactual gel banding due to primer mispriming is potentially reduced. All steps of the TILLING protocol were optimized for use with Ex-Taq polymerase and its reaction buffer. PCR and CJE optimization, therefore, may be required if using a different polymerase. It is suggested that side-by-side comparisons be performed with Ex-Taq when evaluating alternative polymerases. In addition, polymerase errors may increase false positive signals. This is not an issue when screening samples pooled two dimensionally as polymerase errors are random and two-dimensional pooling requires the presence of a replicating signal in precise gel coordinates (see Note 9).

2. Due to the high cost of gene-specific fluorescently end labeled primers, it is advisable to perform trial reactions with unlabeled primers to test for amplification efficiency. A yield of 7–10 ng/μL of final PCR product is required for robust and consistent identification of mutations when pooling samples eightfold. Primer trials can be discontinued when consistent amplification is achieved and failure rates are suitably low to render trials cost ineffective. Universal primer strategies aimed at using IRDye-labeled universal primers to reduce costs have been described (16, 22, 23). Such strategies may require additional optimization such as side-by-side comparisons with gene-specific end-labeled primers.

3. Primers should be designed to produce an amplicon size between 725 and 1,600 bp. Larger amplicons are possible, but different acrylamide mixtures and electrophoresis conditions are needed to improve band resolution at higher molecular weights. Primers are designed using the Primer 3 program with an optimal primer length of 24 bp and maximal primer length of 30 bp to accommodate a higher melting temperature primer that results in less assay background noise. Primer design and target selection can be performed in a single browser using the CODDLe input utility followed by CODDLe (24)

and http://www.proweb.org/input/. This system has been extensively used for public TILLING services and >90% primer success rate has been described when used for *Arabidopsis thaliana* gene targets (25). PCR amplification may be inhibited with amplicons of high GC (>50%). In this case, a final concentration of 3% TMSO or 0.5 M betaine has been used in some cases to improve amplification (B.J. Till, J.L. Cooper, C. Codomo, M. Darlow, C. Burtner, K. Young, L. Comai and S. Henikoff, unpublished). It has also been observed that the ramp time between primer annealing and Taq extension can be important for robust amplification of high GC templates, with faster ramp times producing higher yields (S. Henikoff and C. Codomo, unpublished).

4. Special care should be taken when using primers labeled with IRDye 700 and IRDye 800. When possible, avoid prolonged exposure of labeled primers and PCR products to fluorescent lights. Primer stocks should be diluted to no more than 100 μM, aliquoted, and stored at –80°C. The smallest amount of primer mix is made and then discarded after use. Fluorescent signal can dramatically decrease when using primer stocks that have undergone repeated freeze–thaw cycles. IRDye-labeled primers often produce lower yield in PCR amplifications than unlabeled versions containing the same oligonucleotide sequence. To obtain consistently high PCR product yield, a mixture of both labeled and unlabeled primers is used in the PCR mix. Presumably, unlabeled primers anneal better to genomic templates and are important to increase target molecules in the early steps of amplification to a critical mass for labeled amplification. An alternative two-step PCR method has been described for the amplification of partially degraded genomic samples from *Drosophila melanogaster* (26).

5. Reaction volumes for this protocol are scaled for 96-well assays. Pipetting is aided by the use of multichannel pipettors, and assays are compatible with liquid handling robotics such as Hydra and Apricot microdispensers. Assay volumes are suitable for standard 384-well microtiter plates, master mix volumes can be scaled, and the protocol followed as described. A single person can perform assays using six 96-well plates or four 384-well plates at one time.

6. A crude CJE containing the CEL I enzyme has been used for the discovery of thousands of mutations by the TILLING method. Use of a crude extract from *Brassica* petioles has also been described, as well as the enzyme ENDO1 from *Arabidopsis* (27). The commercially available Surveyor nuclease can be used for TILLING and Ecotilling in addition to S1 nuclease and crude and purified versions of mung bean nuclease (21).

Care should be taken when exploring alternative enzymes for mismatch cleavage as extensive optimization may be required.

7. For rice, we use a concentration of 0.06 ng/μl with samples pooled eightfold in a two-dimensional format (7). The optimal concentration for high-quality TILLING and Ecotilling images depends on many factors including PCR amplification, CJE digestion, and sample purification and therefore can vary from lab to lab. A genomic DNA titration can be performed in a single 96-well assay to determine the optimal amount of genomic DNA for TILLING or Ecotilling (28).

8. The quality and quantification of genomic DNA starting material are crucial for TILLING and Ecotilling applications. A variety of different kits and methods of DNA extraction may be suitable, including the FastDNA kit previously described for *Arabidopsis*, *Drosophila*, maize, rice, and soybean TILLING (7, 25, 26, 29, 30). Extraction and sample quantification methods should be thoroughly tested before deciding on a strategy and scaling-up operations. Accurate DNA quantification is essential when pooling samples prior to screening. Large-scale TILLING projects have relied on simple agarose gel assays for genomic DNA quantification, and this can be considered a standard by which to compare other methods (14, 25, 26).

9. DNA pooling provides higher throughput by allowing less machine time per sample for mutation discovery. To date, the highest pooling used for large-scale TILLING screens of diploid organisms has been eightfold. Studies in *Arabidopsis* have shown that heterozygous mutations representing 1 in 16 in an eightfold pool are recovered at the expected 2:1 ratio compared to homozygous mutations, suggesting that mutation discovery is robust in eightfold pools (31). Both one-dimensional and two-dimensional sample pooling and arraying strategies have been described (14). In one-dimensional pooling, 768 unique plants can be screened in a single mutation screen. Pools where mutations have been identified then need to be deconvolved and DNA from individual plants screened to determine the plant in the original pool harboring the mutation (15). In the two-dimensional strategy, samples are first arrayed in an 8 × 8 grid and then pooled by row and by column. 384 unique samples are screened in a single 96-well assay and a true mutation will produce bands on two lanes of the gel (Fig. 1). Lane coordinates provide a key to determine the exact plant harboring the mutation. While the one-dimensional strategy can offer a higher screening throughput, the two-dimensional strategy provides an independent confirmation of mutations in the same assay, potentially reducing discovery errors. The two-dimensional strategy

also reduces the number of liquid handling steps that can be a source of human error. For these reasons, it is suggested to start TILLING projects with two-dimensional pooling of samples. A one-dimensional strategy can be considered when TILLING or Ecotilling have been thoroughly established and increased throughput is desired.

10. Sample purification removes salts and detergents prior to gel loading and allows for sample volume reduction via evaporation. Sephadex columns provide a rapid and robust method for sample purification, but in theory any method that provides salt removal and allows for volume reduction can be considered. Alcohol precipitation has been described as an alternative method (32).

11. It is important to deposit sample directly over the center of the Sephadex column, thus avoiding any disturbance of the column such as touching the column with pipet tips.

12. When using a multichannel pipettor and the gel loading tray to load membrane combs, gel lane to sample well correspondence can differ depending on how samples are deposited into the tray (e.g. column or row priority, 8 or 12 channel pipettor). Decide on a method and then determine the sample wells that correspond to lanes 4, 12, 20, and so on. A simple guide for the deposition of the 200 bp marker into the proper wells can be created using and old 96 tip box insert. Color the rim of the holes corresponding to the appropriate wells. Prior to deposition of the 200 bp marker, place the guide above or below sample plate, and deposit 200 bp marker following colored circles.

13. Gel plates may be pre-poured and stored for up to several days at 4°C covered in plastic wrap. Each 25-cm plate requires approx 20 mL. To assemble new plates, clean the plates with dilute liquid detergent (i.e., 2% Tween® 20) and a soft scrub brush. Rinse plates with distilled water, wipe down with 0.2 N HCl, rinse with distilled water again, and wipe with isopropyl alcohol. Spacers are cleaned by wiping with a wet tissue. Assemble the pieces with the screws backed off and align the pieces by standing the assembly vertically while tightening the screws. Tighten just beyond where you begin to feel resistance (overtightening will crack plates). Place on a horizontal support. When handling acrylamide or polyacrylamide gels, always wear gloves, as unpolymerized acrylamide is a nerve toxin.

14. It is important that the slot is clean, as any loose acrylamide will inhibit insertion of the comb. For increased visibility of the loading well, insert a background card wedged behind the back plate such that it is centered between the ears of the front plate. Two vertical marks can be made on the card that are a comb width apart and will provide a guide for later inserting the

comb precisely in the middle of the gel, which is necessary so that all of the lanes are scanned.

15. For analysis of two dimensionally pooled samples with the GelBuddy program, row pools and column pools from an 8×8 grid should be in adjacent lanes on the gel (Fig. 1). This can be accomplished by several methods. Samples can be pooled into the sample plate in the described order and then with the use of an adjustable width multichannel pipettor sample can be deposited onto the loading tray such that position A1 on the plate represents lane 3, position B1 equals lane 4, A2 equals lane 9, etc. This can also be achieved without the loading tray by using a specialized comb loading robot (Aviso USA). Alternatively, large volume stock plates of pooled DNA can be prepared and arrayed in such a way that row pools and column pools are adjacent after loading into the sample tray by the method of choice (row or column deposition, single, 8 or 12 channel pipettor). This can be easily standardized and is currently the method of choice for TILLING at the Plant Breeding Unit of the FAO/IAEA Joint Programme. Combs can be preloaded and stored at 4°C; however samples are more stable in formamide loading buffer. It is suggested that combs be used within 2 h of application of sample onto teeth. Membrane combs can be washed and reused by soaking used combs in deionized water for a minimum of 40 min. Allow combs to air-dry before reuse.

16. Gels can be run twice, even after a day. The prerun is necessary if the plate has been moved because it is needed for focusing the lasers. After runs are complete, and a new gel is ready, remove the old gel, pour out the buffer from both reservoirs, and clean plates as described. Be sure that the current is off before touching a buffer chamber.

17. For a 1.5-kb fragment, enter the following LI-COR settings: collect time 4 h 15 min, run at 1,500 V, 40 mA, 40 W, 50°C.

18. Practice this step with used combs. Take special care to avoid bending any teeth. If a tooth sticks to the plate, it may not be possible to save it without moving the comb around and thus losing sample from all teeth.

19. Occasionally, LI-COR gel images look fuzzy with diffuse and ill-defined lanes, for any of the following reasons: (1) urea is not thoroughly rinsed from the well before adding Ficoll; (2) the comb is pushed into acrylamide when loaded; (3) the comb moves laterally once inserted into the well; (4) the upper buffer chamber is filled too rapidly after the comb is inserted; (5) Ficoll is not rinsed out of well after the comb is removed; and (6) Ficoll is rinsed too thoroughly from the well after the comb is removed.

20. Alternative methods for data analysis are available. Prior to the advent of specialized software, TILLING data analysis was performed using PhotoShop (33). Commercial software is available through Softgenetics® (http://www.softgenetics.com/jelMarker_tilling.html).

21. The same bench protocols are used for TILLING and Ecotilling. The main modifications for Ecotilling are in experimental design of sample arraying and in data analysis. For sample arraying, non-pooled samples have been screened for many Ecotilling projects. When screened alone, only heterozygous mutations are discovered. To identify homozygous mutations, an equal amount of wild-type or reference DNA, preferably from a sequenced variety, is added to the test sample prior to PCR and mutation discovery. When the discovery of rare polymorphisms is desired, samples can be pooled and arrayed as in traditional TILLING. Pooling, however, may obscure the proper assignment of common polymorphisms (16). Data analysis for Ecotilling is distinct in that all lanes have inherent information. For example, two samples having no bands in a 1.5 kb gene fragment will be scored as genetically similar for that region. In TILLING, only those lanes with bands (putative induced mutations) are valuable and all other lanes can be disregarded. For Ecotilling, therefore, the proper assignment of failed lanes is crucial. It is also important to properly group bands by molecular weight to ensure accurate genotypic associations. Because of this, data analysis for Ecotilling can be more time consuming than for TILLING. Placement of genetically similar samples in adjacent lanes and the inclusion of technical or biological replicates can facilitate gel analysis. It is therefore advised to perform small-scale tests to evaluate different sample arraying strategies before engaging in a large-scale Ecotilling effort.

22. In gene targets with a high percentage of nucleotide polymorphisms, GelBuddy may erroneously link polymorphisms between the IRDye 700 and IRDye 800 channel images causing errors in banding pattern groupings. Because linkages between the two channels cannot be edited, proper banding patterns can be catalogued by marking bands in only one of the two image channels (B.J. Till, J. Jankowicz-Cieslak, and C. Mba, unpublished).

Acknowledgments

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