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EMAIL - a highly sensitive tool for specific mutation detection in plant improvement programs.

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Abstract. TILLING (Targeting Induced Local Lesions IN Genomes) is a useful tool for discovery of specific point mutations in genes of interest to plant breeders. It employs mismatch cleavage detection using endonucleases, particularly CELI and CELII. During PCR annealing, dsDNA heteroduplexes arise in pooled genomic DNA samples containing one or more Single Nucleotide Polymorphisms (SNP) resulting from, for instance, induced mutation. The cleaved fragments can be distinguished from the larger perfectly-matched homoduplex DNA of the unmutated wild types in the sample of pooled individuals. The ability to efficiently detect individuals with specific mutations within pooled samples provides plant breeders with a powerful screening tool to greatly reduce the numbers of plants requiring phenotypic assessment. Further, it enables geneticists to analyse gene function and associate genotype with phenotype.

Such protocols suffer from limited ability to detect mismatch cleavage signal due to non-specific removal, by the nuclease, of 5' end-labelled termini used in the conventional approach. Mutation detection is further limited by high background characteristic of PCR-based end-labelling mismatch scanning techniques. We showed that as nuclease activity increased, internal signal was maintained while 5' signal decayed. Furthermore, internal labelling improved background. The loss of end-signal constitutes a fundamental problem with the conventional approach to mismatch scanning with CEL nucleases.

A new mismatch scanning assay called 'Endonucleolytic Mutation Analysis by Internal Labelling' (EMAIL), was developed using capillary electrophoresis, involving internal amplicon labelling by PCR incorporation of fluorescently-labelled deoxynucleotides. Multiple mutations amongst allelic pools have been detected when EMAIL was applied with the mismatch nucleases CELI and CELII.

This technique offers greatly increased sensitivity in specific-gene mutant detection in pooled samples, enabling enlarged pool sizes and improving throughput and efficiency. We are investigating the limits of pool sizes to deliver a highly efficient mutation detection and analysis strategy for plant breeders and geneticists.

1. Introduction

Over the past decade, the TILLING technique (Targeting Induced Local Lesions IN Genomes) has proven to be a useful tool for the discovery of specific point mutations in genes of interest to plant breeders and other researchers [1-2]. The approach employs mismatch cleavage detection using

endonucleases, particularly the enzymes CEL I and CEL II. During PCR annealing, heteroduplexes in dsDNA arise in pooled genomic DNA samples which contain one or more Single Nucleotide Polymorphisms (SNP), resulting, for instance, from induced mutation. The cleaved fragments can be distinguished from the larger perfectly-matched homoduplex DNA of the unmutated wild types in the sample of pooled individuals. The ability to efficiently detect individuals with specific mutations from within pools of samples provides plant breeders with a powerful early-screening tool with which to greatly reduce the numbers of plants requiring phenotypic assessment. Further, it enables geneticists to analyse gene function and associate genotype with phenotype.

TILLING was developed for scanning populations harbouring point mutations derived primarily by chemical mutation, typically using ethyl methanesulfonate (EMS) [3], and extended to detection of natural mutants [4-5]. Recently, however, TILLING has also proven useful in scanning gamma-irradiated mutant populations [6].

Mismatch detection protocols suffer from a limited ability to detect mismatch cleavage signal due to non-specific removal, by the nuclease, of 5' end-labelled termini used in the conventional approach. Mutation detection is further limited by high background that is characteristic of PCR-based end-labelling mismatch scanning techniques. By studying the activity of CEL nucleases using amplicon substrates labelled both internally and at each 5' terminus, we showed that as nuclease activity increased, internal signal was maintained while 5' signal decayed. Furthermore, background with internal labelling was improved relative to conventional end-labelling techniques. The loss of end-signal constitutes a fundamental problem with the conventional approach to mismatch scanning with CEL nucleases.

2. An improved technique

A new mismatch scanning assay has been developed using capillary electrophoresis, in which amplicon labelling is achieved by PCR incorporation of fluorescently-labelled deoxynucleotides. We have named this strategy 'Endonucleolytic Mutation Analysis by Internal Labelling' (EMAIL) [7]. Multiple mutations amongst allelic pools have been detected when the EMAIL assay was applied with the mismatch nucleases CEL I and CEL II.

The electropherogram in Fig. 1 shows the effect of internal labelling compared to 5' end-labelling. The upper panel shows a sample pool detected with end-labelling; the lower panel shows the result of internal labelling. The sample comprised a 6-fold genomic DNA pool consisting of 4 homozygous wild-type rice (*Oryza sativa*) individuals but with inclusion of two additional homozygous mutant individuals. The rice samples consisted of cultivars highly characterized for their SNP content in exon 8 of starch synthase IIa. The mutant cultivars were known to contain SNP of A>G (SNP3) and GC>TT (SNP4) at positions 2412 and 2543-2544, respectively [8] (NCBI cDNA accession AF419099).

The electropherogram panels represent different dye traces from a single injection of purified heteroduplex digestion products following 18 min of CEL I activity. The single PCR product was amplified from the genomic DNA pool consisting homozygous wild-type individuals plus the homozygous mutant individuals, SNP3 and SNP4. The PCR product is in one instance internally-labelled with fluorescent dUTP [R110] (6-carboxyrhodamine), and in the other case, end-labelled at both 5'-termini with HEX (hexachloro-6-carboxyfluorescein), represented by the blue and green traces respectively. [R110]-labelled nucleotides were present in the PCR at 4µM. Peaks are noted for each site of mismatch cleavage. In each case, the ~5nt shorter internally-labelled peak (lower panel) is of considerably higher signal strength than its end-labelled counterpart (upper panel). Both cleavage fragments from SNP3, and the larger fragment from SNP4 are detectable, however, detection of the ~100bp cleavage fragment from SNP4 in the end-labelled sample is limited due to significant background in the small size range. In the internally-labelled trace, all cleavage fragments from SNP3 and SNP4 are clearly detectable, both due to increased signal strength and smooth reduced background, especially in the small size range. Although incomplete removal of [R110]dUTP artefacts (see lower panel) has resulted in carry-over, this does not pose significant problems for the purposes of mutation scoring, since the resultant interference is at two single-points, the 'size' of which is

expected at ~76 and ~141 'nt'. For this individual assay, CEL I digestion time has been increased to optimize the internally-labelled approach, however, detection of the ~100nt cleavage fragment may not necessarily be improved simply by reducing digestion time, since increased background in the small size range will also result.

In summary, non-specific digestion of the end-labels results in significantly reduced signal from the cleaved amplicons (upper panel) compared to the result with internally labelled amplicons.

This new technique offers an increased degree of sensitivity in specific-gene mutant detection in pooled samples thereby enabling enlarged pool sizes thus improving throughput and efficiency of the mutation scanning process. We are now investigating the limits of pool sizes in order to deliver a highly efficient mutation detection and analysis strategy for plant breeders and geneticists.

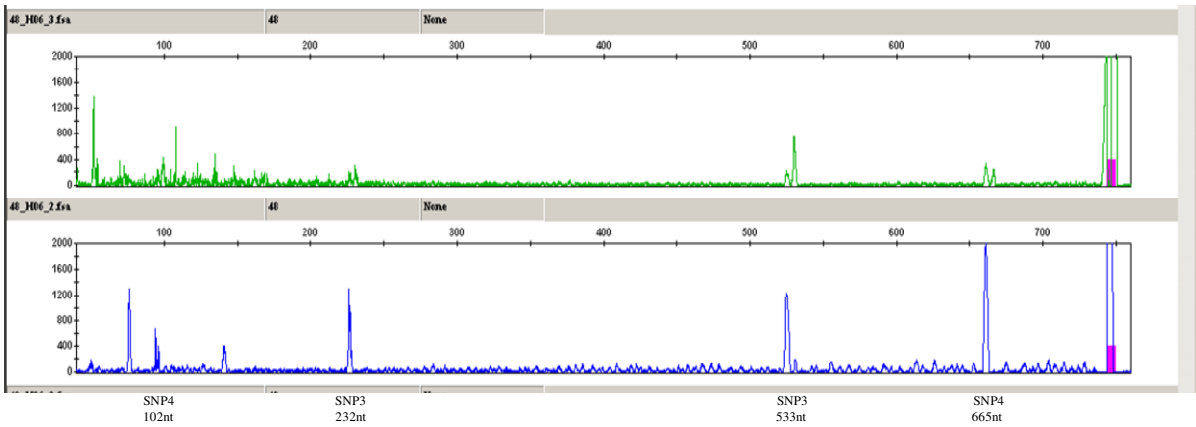

FIG. 1. Comparisons of starch synthase IIa electropherograms of a pooled sample of rice genomic DNA digested with CEL I. The sample included two individuals with SNP mutations, detectable as cleavage fragments. The upper panel shows 5' end-labelled PCR products, whilst the lower panel shows the effect of internal labelling in increasing relative signal strength.

3. Practical implications

Previous work in our laboratories [4] demonstrated the improved detection of point mutations in polyploid sugarcane specimens by taking advantage of the sensitivity of capillary electrophoresis systems. This effectively equated to 14-fold pooling due to the high ploidy level. Our research to date on the EMAIL technology has distinguished discrete SNP individuals in pools of 16 and we anticipate being able to perform routine detection in pools of at least 20. Developments in DNA sequencing have made sequencing the most cost effective method for detection of most common mutations. However, the TILLING approach is an attractive option for discovery of rare alleles or mutations if large pools. EMAIL has now provided a protocol that has the capacity to meet this requirement and may prove to be the method of choice for the detection of rare mutations.

The advent of the EMAIL technique introduces a significant improvement in the efficiency of scanning pools of samples potentially containing point mutations in specific genes of interest. Further, because of the degree of resolution of capillary electrophoresis, information is simultaneously obtained regarding the location of the mutation in the DNA sequence. Accordingly, the technique offers the plant breeder a new tool for efficiently screening induced mutant populations at an early stage for variants in genes of interest before taking plants to field trial. It has the added advantage of providing information to assist in molecular characterisation of mutations in genes of interest.

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