Distilling Gene Function in Barley: A Reverse Genetics Approach.

David Caldwell, Niki McCallum, Sharon Mudie, Gary Muehlbauer, Robbie Waugh
Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5DA, UK

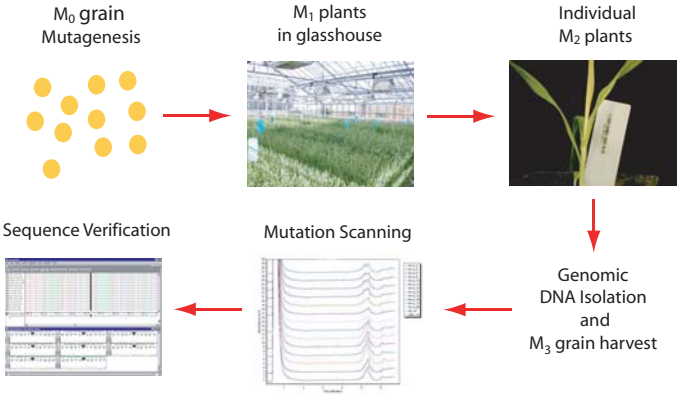
Scottish Crop Research Institute

Introduction

Functional genomics in barley (Hordeum vulgare L.) is a relatively under-developed area of research due to the lack of structured reverse genetics populations. Here, we describe the creation of two large-scale reverse genetics populations derived from Ethyl-methanesulfonate (EMS) mutagenised barley (cv. Optic). Approximately 23,000 M2 plants were obtained from two different treatments of EMS (20mM, 30mM). AFLP analysis of mutation frequency indicates a range of one mutation approximately every 800,000 nucleotides. Genomic DNA has been isolated

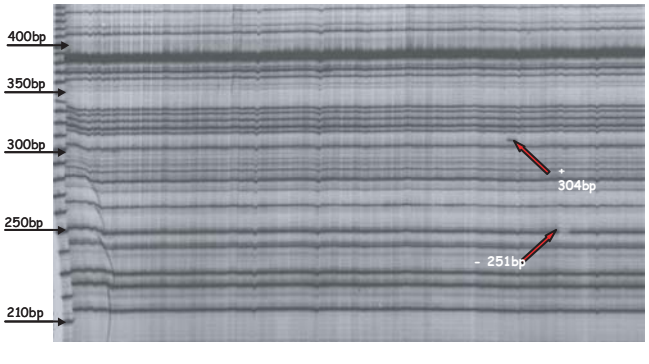
from each of the first 10,000 plants and arrayed into one dimensional pools for identifying mutations in genes of interest. Currently, several mutation scanning approaches can be implemented for screening the population. The Cel I cleavage assay has been successfully used in barley, but is currently limited to pools of 8 plants using an unmodified protocol. Recent advances in dHPLC detectors (Transgenomic, Inc.), has shown the ability to discover SNPs in pools of 48 plants without the use of fluorescently-labeled primers. The multifunctional property (forward & reverse genetics) of this population will be a valuable asset for the worldwide barley research community.

Overview of Creating the EMS Population



M_0 grain was mutagenised with EMS. The mutagenised M_0 grain gave rise to M_1 plants. The M_1 plants yielded M_2 grain. No more that two M_2 grain per one M_1 parent was taken forward, self polinated, and grown to maturity. Young leaf tissue was harvested, genomic DNA isolated from 10,000 plants and the M_3 grain was harvested. Individual M_2 genomic DNA was 1-Dimensionally pooled and stored in 96 well titer plates. Regions of interest will be amplified with gene specific primers and scanned for mutations. Individual M2 lines were identified and mutations confirmed.

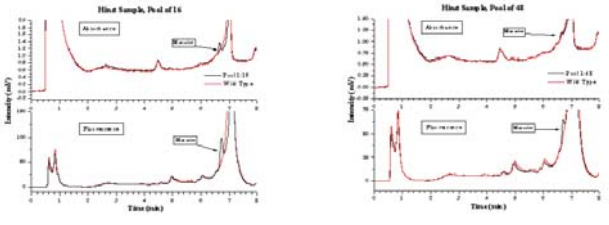
AFLP Mutation Analysis



Mutation frequency in the different M2 populations has been assessed based on lethality, visible phenotypes and a genome-wide mutation scan (using AFLP). As expected, M1 lethality increased with a corresponding increase in mutagen dose. We observed little correlation between lethality in the M1 and M2 generations. The AFLP analysis - while not quantitative - provided an overall 'guestimate' of the relative mutation frequency in the various populations at the level of mutations per base pair. Based on the appearance of new bands or disappearance of existing AFLP bands, frequencies of 1 mutation per 100,000 bp was estimated. Correlation with the 'kill' and visible phenotype frequency was high. These results prompted the development of a large scale "EMS Mutant Population".

Mutation Detection Techniques

A critical aspect in implementing our reverse genetics approach is the choice of a mutation detection method which will allow us to screen PCR-amplified target gene sequences with high sensitivity & specificity as well as low cost. Through a collaboration with Dr. Tony Yeung (Fox Chase Cancer Center) we have investigated the efficacy of enzymatic cleavage of heteroduplex DNAs using CEL I, a mismatch specific endonuclease. The CEL I system is a simple assay that requires PCR amplification of the target sequence, denaturation and annealing to allow formation of heteroduplexes between the wild type and the mutant allele, enzymatic mismatch cleavage, and analysis of the cleaved products by gel electrophoresis. Currently single mutations can be detected in pools of 8 plants (16 alleles).



dHPLC analysis of test pools

Pools of 16 and 48 alleles analysed on a standard UV and the new Fluorescence detector. Please note the difference in signal strength.

In addition, we have investigated the application of denaturing HPLC which is advantageous over other mismatch detection systems as it requires no post amplification template modification, is not gel based, and as a result is both inexpensive and HTP. Through a collaboration with Transgenomic, we have tested their next generation detector. In our test panels the new detector has shown the ability to consistently detect an individual SNP in pools of 48 alleles (24 plants). This increase in pooling depth will greatly reduce the cost and increase the speed of screening the population.

Practical mutation detection

Using the first populations and mutation scanning protocols described above we have now identified mutations in 5 different genes by reverse genetics. Three of these are proprierty and the other two are Hordindoline-a and the barley orthologue of floral organ regulator (flo) from Rice (*Hv*flo). Induced mutations were confirmed by comparison to an extensive haplotype analysis of each of the genes. In contrast to the AFLP analysis the frequency of induced mutations was lower, at around 1:800,000bp, one third that described for Arabidopsis TILLING populatios.
The populations are of course also very useful for forward genetic screens. Phenotypic data from 23,000 10-16 plant M3 families has been scored and entered into a web accessible database that can be remotely queried and seed requests made directly over the internet.

Acknowledgements

- This project is being funded by the Scottish Executive Environment and Rural Affairs Department.

- We would like to thank Dr Tony Yeung for supplying the Cel I enzyme.

- We are very grateful to Transgenomic for allowing us to test their next generation detector. Specifically; Ben Legendre, Dr Mike Marino, Craig Parker.

SCOTTISH EXECUTIVE
Environment and Rural Affairs Department

TRANSGENOMIC
transforming the world