Fast tracking MAS through a new tissue sampling technique – An investigation

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Abstract

Recent developments in molecular marker techniques have allowed the throughput of marker assisted selection (MAS) in breeding programs to increase rapidly over the past few years. This is largely a result of more efficient DNA extraction methods and the fluorescent-based detection of multiplexed PCR markers on semi-automated DNA fragment analysers. To streamline and facilitate the screening of larger populations a more efficient tissue sampling technique and planting strategy is required. Glasshouse space and seed / data management has now become one of the limiting steps in marker screening.

This paper investigates the opportunity to implement a new tissue sampling technique that would alleviate limiting factors such as glasshouse space. Barley seeds are planted, grown and harvested in the laboratory in a 96 well format. The DNA extraction is performed on root tissue following freezing to capture a portion of the root tissue. By harvesting only some of the roots, the seedling is left viable. Hence after MAS, lines carrying the desired alleles can be transplanted in the glasshouse to reach maturity. This technique has been successfully used in Arabidopsis and rice (5).

Key words DNA extraction, tissue sampling

Introduction

Marker assisted selection (MAS) within The University of Adelaide Barley Program focuses on the screening of F_1 derived material, however other analyses such as doubled haploid screening, the identification of introgressed segments in elite lines via graphical genotyping, pure seed confirmation and validation experiments are also routinely performed. Improvements in DNA extraction protocols, the use of robotic pipetting and recently the employment of fluorescent-based detection of multiplexed PCR markers have increased the possibilities for higher throughput. At present approximately 5,000 barley lines are currently screened every year. Further increases in throughput will require the bottle necks of glasshouse space, plant identification and harvesting time to be addressed.

There have been few improvements in harvesting technologies or in improving glasshouse efficiency. Researchers have described harvesting ‘leaf discs’ using a hole punch or the cap from an microcentrifuge tube (2), but these methods are relatively labour intensive and prone to error. A hand-held leaf sample collector, still in the developmental stages has been patented by CHAGA (Centre for High-throughput Agricultural Genetic Analysis)(1). In this system, leaf material is inserted into the front of the sampler and a hole is punched into the leaf, the leaf disc is then automatically placed into the well of a 96- well plate. These sampling strategies give an alternative approach to harvesting but do not provide a solution for minimising glasshouse space or plant identification.

A promising method has been described by Kysan (2004), using an ’ice-capture’ technique whereby plants are grown and harvested in a 96 well format in the laboratory. This technique involves growing root tissue into a plate filled with water and harvesting this tissue via freezing. By harvesting only some of the root tissue, the seedling is still viable allowing selected lines to be replanted for maturation. The successful capture of root tissue and transplantation was reported in both Arabidopsis and rice. Here we look at adapting this method for MAS within The University of Adelaide Barley Program.

Method

The principle of the ice-capture technique is shown in Figure 1. In this method, seeds are grown in a 96 well plate that has holes in the bottom to allow the roots to grow through into a second water filled plate. The root tissue is then 'captured' by freezing the second plate and harvested by separating the plates and removing the root tissue from the seed in a non destructive manner. The procedure described in Figure 1. is adapted from Kryson 2004, and provides optimal conditions for growth and non-destructive harvesting of barley seedlings.

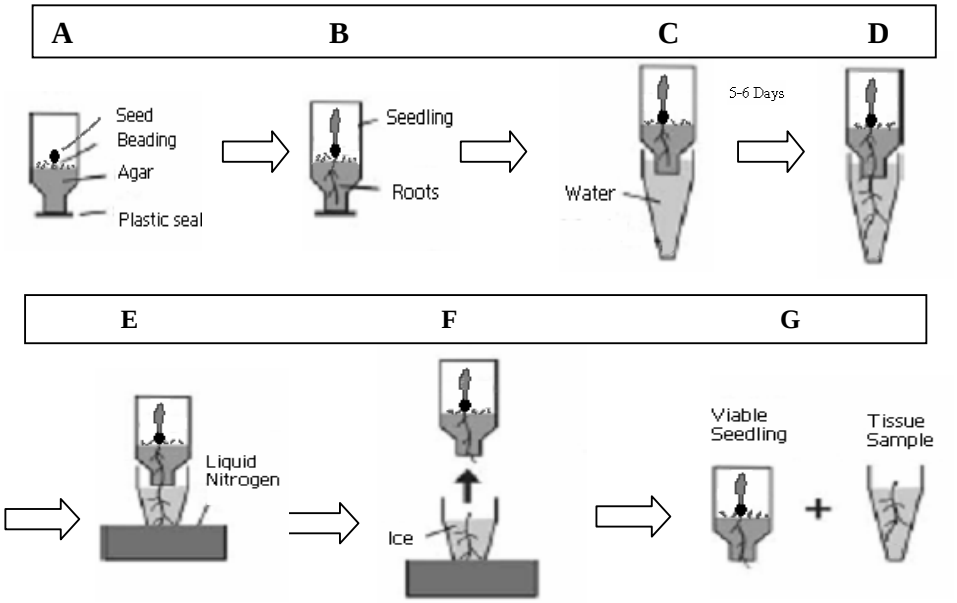


Figure 1. Overview of ice-capture technique.

A. Seeds are pre-germinated on moist filter paper and one seed is placed in each well of a 96-well 1mL unfritted filter plate (Cat # NUN2-780812; Medos) containing 200uL of solidified 0.4% agar topped with 4-6 pieces of hydroponic beads.

B. A seal is placed on top with holes punched through to aerate each well, the plate is then incubated in the dark at 20°C for 2 days.

C. After the seed has germinated to a stage where the roots have penetrated the agar, a 96 well 'chimney stack' plate (Cat # I3410-00; Astral Scientific) is filled with 200uL of water and placed underneath the unfritted filter plate.

D. The seedlings are grown for a further 5-6 days to allow the roots to penetrate into the lower plate.

E. The chimney plate portion is placed in liquid nitrogen to freeze the water containing the root tissue. It is important to ensure that the level of liquid nitrogen freezes only the bottom chimney stacked plate, and leaves the top unfritted plate at room temperature.

F. The chimney stacked plate containing the frozen root material is then peeled away from the unfritted filter plate.

G. This yields a 96 well plate containing the seedling and a plate containing frozen roots which can then be used directly for DNA extraction. The harvested top unfritted filter plate containing the seedlings, is then placed into a fresh 96 welled plate filled with water to keep seedlings hydrated before transplanting.

Results and Discussion

Currently the planting and harvesting of F_1 seed is a time-consuming process requiring time to pot soil, plant seedlings, label pots, harvest leaf tissue, grow plants to maturity and harvest seeds. The labour, consumables and glasshouse space required is substantial. The adoption of the ice-capture method will provide an opportunity to eliminate many of the time limiting steps of glasshouse harvesting. By growing seeds initially in a 96 well format, only those plants which carry the desired alleles need to be planted. This saves on consumables (pots and soil) glasshouse space and time. In addition individual plants do not require labelling as they will be identified by positions in the 96 well plate. It is also anticipated that this process will reduce human error in tissue harvesting as the material for DNA extraction is already in the correct format. Our preliminary work has focused on establishing if this method is suitable for use in barley, but is expected to be broadly applicable to winter cereals.

Growth of seedlings, harvesting of tissue and transplantation

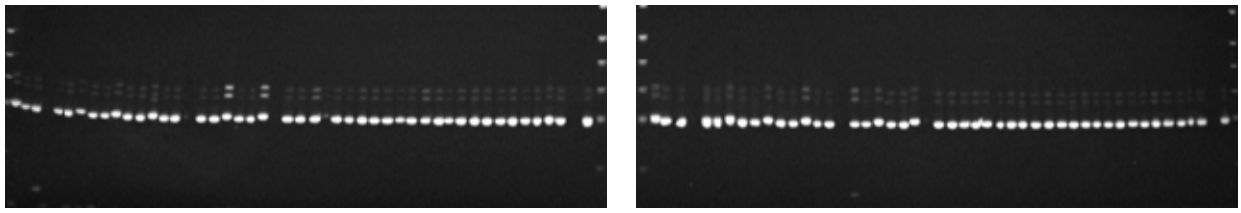
It was necessary to modify several steps of the method described by Kryson (2004). The problems encountered were mainly associated with achieving satisfactory germination of seed and root growth. We found it was important to provide adequate aeration to prevent the seed from becoming 'water logged'. To avoid this, hydroponic beads were added to the top of the agar and holes were punched in the PCR seal used to cover the unfritted filter plate. We also had much better success using pre-germinated seed. Several replicated experiments showed a 76% germination rate with un-germinated seed compared with 94% for pre-germinated seed. Experiments also compared the effect of adding water to the unfritted plate (Step A), the amount of agar added to the unfritted plate (Step A), and the time seed was left before harvesting (Step D). Optimal results were obtained when 80uL of water was added at Step A, 200uL of agar was used in each well of the unfritted plate and the seed was left for 5 days before harvesting.

In addition to ensuring the successful germination and growth of seeds it was also vital that the seedlings survived when transplanted to soil. This was confirmed by transplanting 48 seedlings to soil. In this instance, all but two plants survived. In addition harvested seedlings were successfully transplanted after being store in refrigerator for two weeks in the unfritted filter plate. This demonstrated that the ice capture method would allow marker assisted selection to be performed whilst seedlings were stored in the unfritted plates. This provides enormous time and space savings as only seedlings having favourable allelic combinations need to be transplanted.

The only major change to the original ice capture protocol related to the process of harvesting the roots material. Kryson (2004), used an ethanol / ice bath, however we had little success with this process and found that the water in the chimney stack plate did not sufficiently freeze to allow the plates to be 'peeled apart'. Consequently, we have used liquid nitrogen, which froze the water completely and quickly.

DNA extraction and genotyping

The method described by Kryson (2004) for extracting DNA from roots involves the use of ball bearings to crush the material and the addition of Tris and EDTA. We had little success with this protocol and alternatively adapted our current high-thoughput NaOH DNA extraction procedure described by (3). To confirm that DNA could be successfully extracted from the harvested root tissue using the ice-capture method, the quality of DNA extracted by NaOH was compared with root and leaf tissue obtained from the same samples. Both root and leaf tissue, were exposed to identical methods of microsatellite amplification (3). For the samples that germinated (spaces in the gel are from seeds which failed to geminate, not failed PCR), we observed 100% amplification from DNA samples isolated from root and leaf tissue (Figure 2.). In addition the DNA samples were suitable for the multiplex ready PCR assay (4). Figure 3. shows fluorescent based microsatellite amplification. Genomic DNA samples extracted using three methods, specifically, phenol chloroform from leaf material, NaOH extraction from ice-cap harvested root tissue and NaOH extraction from leaf.



(a) **(b)**

Figure 2. Comparison between (a) leaf tissue and (b) root tissue extracted using the NaOH extraction method. DNA samples were amplified using the microsatellite marker GBM1438.

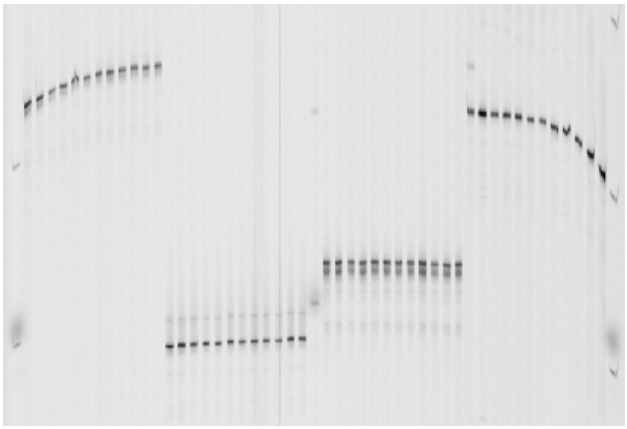


Figure 3. Multiplex-ready PCR amplification of four flourescent SSR's amplified from DNA samples prepared using three extraction methods. Starting from the top left lane, Lane 1&2 phenol chloroform extraction from leaf material. Lanes 3-7 NaOH from ice-cap harvested root material. Lanes 8-12 NaOH from leaf material. All tissue samples were of the variety Sloop.

Conclusion and Future Directions

The investigation into the adoption of this ice capture technique is still in the preliminary stages. We have successfully adapted the technique for the growth and extraction of barley rootlets. However, further experimentation at the rootlet growth stage, harvesting stage, seedling survival time in plates and success of transplantation is needed to completely assess the merits of this technique.

The results presented demonstrate that the ice-cap technique can be used to harvest root tissue for use in our NaOH extraction technique. This extraction method yields DNA which can be used in multiplex ready PCR (4) as well as with conventional PCR assays.

There are numerous benefits to adopting this technique for MAS. It provides an opportunity to overcome the current bottleneck which exists in the planting, identification and subsequent harvesting of plant material. Moving, the planting and harvesting process into the laboratory will increase genotyping throughput and allows more efficient management of large screening populations.

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