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HIGH-THROUGHPUT GENOTYPING OF CEREALS

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INTRODUCTION

Correct identification of cereal variety is vital for quality assurance of goods requiring different grain attributes. In barley for example, the malting and brewing properties are variety dependent, thus sourcing the correct variety is critical to product consistency. It is becoming increasingly important to be able to identify cultivars rapidly and cheaply to determine purity or levels of admixture in grain samples, in order to make the most appropriate use of agronomic types available and/or quality characteristics. In order to achieve this, a molecular marker and genotyping platform suitable for screening large numbers of individuals must be found.

Single nucleotide polymorphisms (SNPs) are changes to a single base at discrete loci in the genome and are generally bi-allelic (Gut, 2001). The frequency of SNP markers in the barley genome, approximately 1 per 130 to 200bp (Bundock *et al.*, 2003; Kanazin *et al.*, 2002; Rostoks, Mudie *et al.*, 2005) makes SNPs an attractive genotyping marker. *In silico* SNP discovery is inexpensive and while whole genome data is currently not available for large-genome species such as barley and wheat, large numbers of putative SNPs can be screened from EST-derived unigene databases (Rostoks, Borevitz *et al.*, 2005).

Demand for high throughput, cost-effective genotyping has driven the development of commercial genotyping platforms capable of typing thousands of SNPs from large numbers of individuals. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) provides one of the most powerful and reliable methods for high-throughput SNP genotyping (Buetow *et al.*, 2001). We used Sequenom[®] MALDI-TOF mass spectrometry to develop a rapid, high throughput SNP identification assay capable of determining barley variety identity and purity from Australian commercially grown barley cultivars with precision and speed.

MATERIALS AND METHODS

Sample Collection and DNA extraction

A collection of barley (*Hordeum vulgare*) germplasm representing 55 malting and feed cultivars of significance to the Australian barley industry was obtained from the Australian Winter Cereals Collection (AWCC), Tamworth (NSW, Australia). DNA was extracted from leaves of each individual approximately 10-14 days post-germination from a minimum of 10 individual seedlings using a Qiagen MagAttract DNA extraction kit as per manufacturers instructions. The DNA was stored at -20°C.

SNP Identification

SNP sites in 31 barley genes were used for the development of the MALDI-TOF assay. SNPs were identified by aligning barley expressed sequence tags from public databases. The targeted genes form the basis of allele-specific PCRs and correspond to cDNAs used as restriction fragment length polymorphic probes for linkage

mapping in barley (Bundock *et al.*, 2006). For each candidate SNP, flanking primers and an extend primer were designed using MassARRAY Assay Design™ software (Sequenom Inc.) and synthesized. Assays were designed for uniplex and multiplex applications. Genomic DNA (2ng) was PCR amplified using 0.5U HotStar Taq DNA polymerase (Qiagen), 0.1µM each flanking primer, 500µM each dNTP, 1.625mM MgCl₂ and 1.25x PCR Buffer (supplied with enzyme including 15mM MgCl₂) in 5µl. Thermocycling conditions were as follows: 94°C for 15 min, 45 cycles of 94°C for 20 sec, 56°C for 30 sec, 72°C for 1 min, with a final extension step at 72°C for 3min.

Genotyping

Following PCR amplification of the target, reactions were incubated with shrimp alkaline phosphatase (SAP) to neutralize unincorporated dNTPs. The SAP enzyme solution consisted of 0.3µl SAP enzyme (1U/µl), 0.17µl of 10x SAP Buffer (supplied with Sequenom iPLEX™ enzyme) in 2µl. Reactions were incubated at 37°C for 20 min, 85°C for 5 min, then held at 4°C. After SAP incubation, reactions were mixed with 2µl of the iPLEX™ (Sequenom, Inc.) cocktail (1x iPLEX enzyme, 1x iPLEX termination mix and 0.222X iPLEX buffer, 0.625µM extend primer) was added to each reaction bringing the total volume to 9µl. The reaction was thermocycled as follows: 94°C for 30 sec, 45 cycles of 94°C for 5 sec, (5 cycles of 52°C for 5 sec, 80°C for 5 sec); followed by a final extension step at 72°C for 3 min. Next, 25µl water was added to the reactions, followed by addition of Clean Resin™ and mixed by gentle inversion for 15 minutes. Finally, iPLEX reaction products were spotted onto a SpectroCHIP™ for MS analysis, using the MassARRAY Nanodispenser.

The assay was carried out initially in uniplex on a DNA set consisting of one individual per cultivar. The assay was then validated in multiplex on 10 to 25 individuals from each cultivar.

RESULTS

SNPs identified *in silico* were used to design a MALDI-TOF assay. The uniplex assay was successful in identifying homozygous and heterozygous alleles from 31 genes in 55 cultivars of barley. The data was pooled to build a panel of SNPs unique to each cultivar. Further work is continuing in order to identify multiplex capacity and quantification of mixtures of cultivars.

DISCUSSION

Rapid advances in SNP discovery are providing vast amounts of data, ready for detection and diagnostic applications. The priority now is developing cost-effective platforms capable of rapidly and accurately identifying genetic polymorphisms (Shi, 2001), combined with multiplex and automated high-throughput detection capabilities. The challenge for HTP plant genotyping lies in utilizing the available data more effectively, and improving the capacity and cost-effectiveness of screening thousands of polymorphisms from large numbers of individuals. The greatest obstacle is the affordability of testing and validating novel HTP genotyping assays.

The advantage of MALDI-TOF MS genotyping lies in the direct identification of SNPs by determining the mass of the polymorphic nucleotide itself. Genotype calling was precise and coupled with the ability to multiplex up to 25 reactions per well on a 384-well plate in a fully automated process, MALDI-TOF mass spectrometry clearly

has the potential to be a high-throughput genotyping method of choice for cereal identification.

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