9 Nanotechnology: The Future of Cost-effective Plant Genotyping

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Introduction

The exponential growth in biotechnology and nanotechnology industries today along with associated decreasing costs can be compared with that of the silicon chip revolution. Gordon Moore, a co-founder of Intel, is often quoted as having said in 1965 that computer processing power would double every 18 months, an observation now known as Moore's law. Stated correctly, however, Moore said that the complexity for minimum component costs increased at a rate of roughly a factor of two per year. That is, cost per unit could be reduced by increasing the number of features per unit area in addition to decreasing unit size. Moore's observations in 1965 led him to speculate quite accurately on the impact of inexpensive computing power on the way we go about our daily lives today.

So what has the silicon revolution got to do with genotyping molecular markers in plants? While production and commercialization of transgenic plants has been relatively successful, molecular marker technology for plant breeding has not been adopted as swiftly (Gupta *et al*., 2001). Factors affecting whether and how molecular markers should be used in plant breeding programmes include cost of the technology, cost of measuring the trait and sample turnaround time (Lamkey and Lee, 1993). Large segregating populations are difficult to manage and cost-effective, high-throughput (HTP) approaches to date have not been available, thus restricting wider implementation of molecular marker technology in plant breeding (Gupta *et al*., 2001). Ultimately, in order for molecular marker technology to become more widely adopted, reducing the cost of interrogating a data point for the purposes of identifying an allele, trait or individual remains the challenge.

The scope of this chapter includes a review of first and second generation molecular marker genotyping techniques with the focus on single nucleotide polymorphism (SNP) detection. Using barley (*Hordeum vulgare* L.) as a

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case study, we identify the useful application of these genotyping techniques and discuss the limitations of these to HTP genotyping applications. Nanotechnology and a number of nanoparticle-based assays are subsequently explored and the potential cost-effective implications of this third generation genotyping technology on multiplexing and HTP genotyping are discussed.

Genotyping Single Nucleotide Polymorphisms in Barley

Barley is an important crop used for both food and beer-making. As a species, barley is extremely variable and is cultivated in a wide range of environments (Kanazin *et al*., 2002). In plant breeding terms, the capacity to generate genome-wide molecular markers rapidly and easily would constitute a significant improvement to quantitative trait loci (QTL) analysis, marker-assisted selection (MAS) and variety identification (Kanazin *et al*., 2002). Correct identification of barley varieties is imperative for controlling the quality of goods requiring different grain attributes, for example, beer-making and animal feed, and to ensure the best use of agronomic genotypes available (McCausland and Wrigley, 1977). Verification of genotype may also be necessary to ensure that royalties on improved varieties are paid to plant breeders, thus providing more resources for further plant improvement. Thirty years ago, molecular marker identification of barley varieties involved starch gel electrophoresis and isoelectric focusing, in combination with the phenotypic marker aleurone colour (McCausland and Wrigley, 1977). In the three decades since, significant quantities of molecular marker data have been generated for barley using a variety of methodologies including PCR (Chiapparino *et al*., 2004; Bundock *et al*., 2006), sequencing (Kanazin *et al*., 2002; Bundock *et al*., 2003; Bundock and Henry, 2004; Russell *et al*., 2004), high-performance liquid chromatography (HPLC; Kota *et al*., 2001b), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Paris *et al*., 2002, 2003) and *in silico* SNP discovery from expressed sequence tag (EST) databases (Kota *et al*., 2001a, 2003). SNPs are loci where the sequence of DNA differs by a single base and are ideal for genotyping as they are the most abundant, stable marker in both animal and plant genomes. Estimates of the frequency of barley SNP markers vary from around 1/130 to 1/200 bases (Kanazin *et al*., 2002; Bundock *et al*., 2003; Rostoks *et al*., 2005b), likely due to differences in the region targeted for analysis. Regardless, these estimates are significantly more frequent than the estimated average for plant genomes of two SNPs per kilobase (Paris *et al*., 2003) and one SNP per kilobase in humans (Wang *et al*., 1998).

SNPs are found in both coding and non-coding regions of the genome and are rapidly becoming the molecular marker of choice for individual genotyping applications. Not all SNPs are useful markers; however, those found in cDNA and promoter region sequence alignments can directly affect gene function and are therefore considered 'perfect' molecular markers (Paris *et al*., 2003). In theory a SNP site could involve four possible alleles, although in

practice SNPs are biallelic, i.e. only two are generally observed at a specific site in a population (Gupta *et al*., 2001). Many methods are available for SNP genotyping, and the choice depends mainly on the scale of the study and the scientific question that is being addressed, i.e. whether large numbers of SNPs are required from a small number of individuals or vice versa (Gut, 2001).

All current methods of SNP genotyping involve generation of an allele-specific product followed by allele interrogation (Tost and Gut, 2002). Broadly speaking, SNP genotyping can be divided into gel-based and non-gel-based methods. The following sections briefly outline the most widely used allele identification methods. However, it is by no means exhaustive, and is intended to highlight the pros and cons of various SNP genotyping methodologies used in plant genotyping, past and present, in order to set the scene for introduction to potential applications of nanotechnology in plant genotyping. For more comprehensive information on SNPs in genotyping and epidemiology see Schork *et al*. (2000); for SNP genotyping in plant systems, see Gupta *et al*. (2001).

First Generation Assays (Gel-based)

First generation molecular markers can be divided into probe-based and PCR-based assays. Probe-based assays include restriction fragment length polymorphisms (RFLPs) and minisatellites. RFLP patterns are generated by cutting genomic DNA with restriction enzymes and polymorphisms occur by the gain or loss of a restriction site. Apart from being time-consuming, the main disadvantage of RFLPs is that the marker must be found in a restriction cutting site. Minisatellites are tandem arrays of short repeated sequences which are found throughout the genome (Jones *et al*., 1997). Polymorphic loci are generated by variable numbers of repeat units and multi-allelic forms (Jones *et al*., 1997). Probe-based assays have generally been time-consuming and also require radioactive labelling.

PCR amplification techniques are varied and include whole genome or targeted allele approaches. Random amplified polymorphic DNA (RAPD) (Williams *et al*., 1990) uses short arbitrary oligomers to prime random sites within the genome producing amplicons between 200 and 2000 kb long (Jones *et al*., 1997). Sequence polymorphisms at priming sites in the genome generate variation between banding patterns. RAPDs are inexpensive and easy to produce; however, they have limitations which have restricted their use including amplification of impurities, marker anonymity and the sensitivity of marker reproducibility to reaction conditions (Bachman, 1994).

Amplified fragment length polymorphism (AFLP) (Vos *et al*., 1995) involves digesting genomic DNA, ligation of the fragments to oligonucleotide adapters and selective PCR amplification of the fragments followed by gel analysis. Denaturing polyacrylamide gels provides high resolution of up to 100 restriction fragments, providing a powerful DNA fingerprinting technique (Vos *et al*., 1995). However, the methodology is time-consuming and requires the use of acrylamide, which is a dangerous neurological toxin.

Allele-specific PCR (AS-PCR) is an inexpensive, targeted approach where the identity of the amplified locus is generally predetermined by sequence analysis. AS-PCR specificity is influenced by the nature of the polymorphism and the flanking sequences. Guanine cytosine (GC)-rich loci make AS-PCR assays and multiplexing difficult to optimize (Shi *et al*., 1999).

Single-stranded conformation polymorphism (SSCP) is an inexpensive, sensitive method for screening PCR amplicons for novel polymorphisms. Under appropriate conditions, single-stranded DNA folds into sequence-specific structures which affects electrophoresis migration rate. A single base change can alter the structure, thus affecting mobility rate through the gel. The sensitivity of the assay is inversely proportional to the fragment size, and thus smaller fragments are preferable (Sunnucks *et al*., 2000). This method is useful for SNP discovery; however, it does not provide any other information except presence or absence of sequence variation between two samples. Once a putative polymorphic locus is found, it must be sequenced to confirm the presence and identity of the allele.

First generation marker technologies have had an enormous impact on the field of molecular biology and gel-based assays are generally facile for small numbers of samples; however, probe and PCR-based methods are not amenable to HTP applications because the assays are time-consuming, labour-intensive and expensive (Bassam *et al*., 1996). As a result, non-gel-based genotyping technologies have emerged as the dominant genotyping platforms for HTP, particularly in the field of pharmacogenetics (Shi, 2001).

Second Generation Assays (Non-gel-based)

Real-time PCR and fluorescent SNP genotyping

Real-time or quantitative PCR (qPCR) monitors the accumulation of target amplicons via fluorescently labelled probes during the reaction. Hence, while only the end point of a standard PCR is assessed via electrophoresis, qPCR enables the user to monitor lag, exponential and plateau phases as they occur. Quantitative PCR and fluorescent SNP genotyping generally involves simultaneous PCR amplification of the target and detection via one or more fluorescently tagged oligonucleotide probes specific to the allele of interest. Probe/target hybridization events generated during each PCR cycle result in an exponential increase in fluorescence. Prior to hybridization with the target, probes are rendered non-fluorescent via contact quenching or fluorescence resonance energy transfer (FRET) quenching. Contact quenching occurs when the reporter fluorophore is brought into direct contact with the quenching fluorophore by secondary folding of the oligo, for example, molecular beacons and Scorpion probes. Upon hybridization to the target, probes undergo conformational change resulting in spatial separation of the reporter and quencher producing fluorescence. FRET quenching occurs when the emission of the reporter fluorophore is altered by the absorption spectrum of a quenching fluorophore in close proximity on the same oligonucleotide, for

example TaqMan probes. On hybridization to the target, a TaqMan probe is cleaved by 5'-exonuclease activity of Taq DNA polymerase, resulting in the irreversible separation of reporter and quencher producing fluorescence.

Molecular beacons are capable of distinguishing targets that differ by a single nucleotide, are more specific than linear oligonucleotide probes (Bonnet *et al*., 1999) and have been successfully exploited for detecting SNPs and transgenes in barley (Kota *et al*., 1999). TaqMan assays have been used extensively in barley pathogen studies, in particular detection and quantification of toxin-producing *Fusarium* spp. (Strausbaugh *et al*., 2005; Leisova *et al*., 2006; Sarlin *et al*., 2006). Fluorescent assays are not suitable for large-scale multiplexing but are amenable to HTP when used to detect a small number of targets from a large number of samples. Fluorescent oligonucleotide probes may be expensive per data point, depending on the amount of oligo modification and while TaqMan chemistry is supported by dedicated software, a significant amount of optimization is required for each of these assays. The major advantage of qPCR over standard PCR includes the homogeneous or 'closed-tube' format, reducing the chance of cross-contamination, elimination of electrophoresis, dual specificity of primers and probes and, significantly, the ability to quantify the target.

Sequencing

Sequencing remains the most reliable method for detecting sequence mutations. The cost of sequencing has decreased over the past decade due to a number of factors including the replacement of gel-based sequencing with automated fluorescent capillary sequencers, improvements in sequencing chemistries and availability of lower-cost HTP sequencers.

The Sanger dideoxy chain terminator sequencing assay (Sanger *et al*., 1977) has been widely used for nearly three decades and capillary sequencers are capable of sequencing up to 700 bases from 96 DNA templates in a single 1-h run. To date, Sanger sequencing has been the main generator of sequence information; however, the requirement for cloning, amplification and purification of individual templates has been a limiting factor to cost-effective, HTP sequencing (Margulies *et al*., 2005).

Pyrosequencing (Ronaghi *et al*., 1996) relies on the conversion of pyrophosphates (PPi) to adenosine triphosphates (ATP) stimulating the light-producing luciferase in a base-dependent reaction. A primer is designed to hybridize adjacent to the 5'-side of the SNP position and all possible variants can be determined in a single-tube reaction as the region is sequenced (Fakhrai-Rad *et al*., 2002). Homozygous and heterozygous variants each give a unique pattern (pyrogram) compared to the wild-type (Ronaghi *et al*., 1996; Ahmadian *et al*., 2000). SNPs can be directly scored without the need for post-sequence editing and multiplexing can be applied to this technique (Fakhrai-Rad *et al*., 2002). Ninety-six-well and 384-well plate instruments are currently available facilitating HTP analysis of 5000–50,000 SNPs per day (Fakhrai-Rad *et al*., 2002). Pyrosequencing has been employed to identify cereal species

including barley (McIntosh *et al*., 2005) and to rapidly classify β-amylase allelic variation in barley (Polakova *et al*., 2003). Pyrosequencing is quickly emerging as an attractive platform for SNP analysis as the technology is time- and cost-competitive (approximately US$0.20 per sample); however, when compared to other genotyping platforms, the cost per data point is still a matter of concern, primarily due to the cost of biotinylated primers and specialized, dedicated instrumentation (Fakhrai-Rad *et al*., 2002).

Recently, a novel method of sequencing, '454 sequencing' (Margulies *et al*., 2005), was described which could potentially sequence 25 million bases in a 4-h run, generating a 100-fold increase in throughput over current Sanger sequencing technology. The 454 sequencing is PPi-based sequencing in picolitre-sized wells. Genomic DNA is isolated, mechanically sheared into small fragments and ligated to adapters. A single fragment is captured on to a bead and the DNA is clonally amplified on the bead, within a droplet in an emulsion (Margulies *et al*., 2005). Beads bearing single-stranded DNA clones are distributed into wells of a fibre-optic slide (PicoTitre-Plate) and the DNA is sequenced by pyrosequencing (Margulies *et al*., 2005). The method was shown to be extremely effective on compact microbial genomes (Margulies *et al*., 2005). However, due to short read lengths of 454 sequences, it was unknown how 454 sequencing would perform on large, highly repetitive genomes such as those of wheat and barley. The 454 sequencing was compared directly with Sanger sequencing on barley Bacterial Artificial Chromosome (BAC) clones, and while repetitive regions were problematic, deep 454 sequencing provided more even coverage of the BAC clones than did Sanger sequencing. A sequencing strategy incorporating some Sanger sequencing to partly compensate for the short read length of 454 sequences was developed by Wicker *et al*. (2006). The authors also concluded that due to difficulties encountered in repetitive regions, a whole-genome shotgun approach may not be practical for very large plant genomes; however, they suggested that small pools of BACs may be more practical for 454 sequencing (Wicker *et al*., 2006). Perhaps the greatest advantages of 454 sequencing over Sanger sequencing include direct sequencing from genomic template and reduced time and labour inputs, for example, 20 mb of sequences can be obtained in 4 h in a single 454-sequencing run (Margulies *et al*., 2005; Wicker *et al*., 2006). In combination with targeted Sanger sequencing, 454 sequencing promises rapid and cost-effective sequencing of coding sections of large, complex genomes (Wicker *et al*., 2006), providing unprecedented acceleration of SNP discovery and genotyping.

Sequencing provides a reliable genotyping method for barley as studies have shown that SNP haplotype is congruent with barley germplasm group (Kanazin *et al*., 2002). Bundock and Henry (2004) used sequencing to determine SNP haplotype diversity of the *Isa* gene of barley. The *Isa* gene is putatively involved in pathogen defence in the seed, and while cultivated barley varieties were less diverse at the *Isa* loci than wild barley (*H. spontaneum*), evidence of nine recombination events in cultivated varieties suggested a recombination hot spot driven by selection (Bundock and Henry, 2004). Genetic diversity and SNP haplotype were then determined from *Isa* sequences of eight wild barley populations from Israel (Cronin *et al*., 2007). Genetic diversity

was significantly correlated to key environmental water variables and genetic diversity at the *Isa* loci in wild accessions of barley from arid regions was significantly higher compared with those accessions from wetter climates. It was concluded that the higher diversity of *Isa* defence proteins observed may be a result of selection pressure by more diverse microbial populations present in arid environments (Cronin *et al*., 2007). Gene diversity linked to agronomically important traits such as plant defence mechanisms is of enormous potential and importance to plant breeding programmes. SNP discovery and genotyping by sequencing has clearly demonstrated capacity to identify potentially valuable loci in barley research programmes.

DNA chips/microarrays/high-density oligonucleotide arrays

The basic principle of 'DNA chips' or microarrays is a binding or hybridization assay (Bier and Kleinjung, 2001). 'DNA chip' refers to miniaturized arrays of nucleic acid oligomers (or probes) immobilized on a flat solid support, usually a glass slide (Hacia and Collins, 1999). Applications of microarrays range from SNP detection and scoring, gene expression to mutation analysis of large genes (Hacia and Collins, 1999; Bier and Kleinjung, 2001). The target is PCR amplified and fluorescently labelled before incubation on the array. After incubation, the array is read by exciting the fluorescent signal with a laser by scanning each spot or imaging the entire array. Thousands of probes can be either synthesized on to the chip during manufacture (Affymetrix, California) or, alternatively, spotted on to the chip by robot after synthesis.

The microarray technique has facilitated multiple measurements of many hybridization events to be performed in parallel, reducing labour and reagent costs, and is readily automated (Bier and Kleinjung, 2001). Oligonucleotide arrays were used extensively in large-scale identification and genotyping of SNPs in the human genome (Wang *et al*., 1998). In 2003, the Barley GeneChip (Affymetrix) was released as the culmination of worldwide collaboration among the barley research community (Close *et al*., 2004). Over 21,000 genes are represented on the array, generated from more than 84 libraries worldwide (Close *et al*., 2004). The chip has potential applications in analysis of malting properties, pest and disease control, abiotic stress tolerance, nutritional characteristics and reproductive development.

Some limitations to microarrays, however, need to be understood for their astute use, particularly in the field of SNP discrimination. Microarrays are primarily a screening tool and their major limitation is low sensitivity to rare events and sequences in trace populations (Hacia and Collins, 1999; Bunney *et al*., 2003). For a non-exhaustive SNP screen where an error rate of 5–10% is acceptable, microarray assays for heterozygous sequence changes are quite useful; however, for more specific applications it is crucial to define the sensitivity and specificity of DNA chip assays beforehand (Hacia and Collins, 1999).

Hybridization efficiency of microarrays is influenced by sample concentration, size and density of probes and melting temperature of each probe.

Each bound probe has its own sequence-dependent melting temperature; thus, it is difficult to ensure homogeneous hybridization conditions for all probes across an array (Bier and Kleinjung, 2001). In its current methodology, discrimination of SNPs at array level may require extremely stringent washes, which could result in the loss of much information (Bier and Kleinjung, 2001). A new approach to discriminate SNP mismatches on microarrays may be to observe the dissociation kinetics during the detection process. Dissociation is much more rapid when mismatches are present and kinetic analysis may be a more accurate method to discriminate mismatches from wild types (Bier and Kleinjung, 2001).

Microarrays have had an important impact on the capacity of DNA screening applications; however, due to their two-dimensional nature and limited pixel size, their use is limited to libraries of 5×10^5 compounds (Trau and Battersby, 2001; Battersby *et al*., 2002). The identity of each individual spot is determined by its position on the grid and high-cost devices are required to keep track of each position (Trau and Battersby, 2001). While microarrays are a highly automated, established platform, the cost per data point must become more economical (Battersby *et al*., 2001).

Minisequencing

Minisequencing is a microarray-based approach to screening for all possible alleles. Probes, designed to hybridize adjacent to a SNP site in the target, are immobilized on a surface at the 5' end leaving an exposed 3'-OH group. The hybridized target serves as template, while the probe acts as a primer for extension with labelled dideoxynucleotide triphosphates. The allele is thus identified by the incorporated ddNTP (Hacia and Collins, 1999).

MALDI-TOF Mass Spectrometry

MALDI-TOF MS is a powerful and reliable tool for HTP SNP genotyping (Tost and Gut, 2002). Paris *et al*. (2003) used MALDI-TOF MS to identify a barley genome SNP which is possibly responsible for barley resistance to powdery mildew. This technology has also facilitated selection for superior alleles of β-amylase, a key starch-degrading enzyme in the malting process, in barley during the seedling stage (Paris *et al*., 2002).

A complete SNP genotyping package including Assay Design software, iPLEX chemistry, liquid handling, chip nanodispensing, MALDI-TOF MS and data analysis is provided by Sequenom. The homogeneous assay consists of PCR amplification of the target, followed by incubation with shrimp alkaline phosphatase (SAP) to inactivate extraneous nucleotides. An 'extend' primer is added, extended through the SNP by a single ddNTP under standard thermocycling conditions, followed by reaction termination. The mass of the primer extension product is determined by laser ionization and desorption of the extension product to determine the sequence of the nucleotide at the SNP site.

MALDI-TOF analysis is highly suitable for HTP SNP analysis and multiplexing because it is fast – ionization and detection take milliseconds (Griffin and Smith, 2000; Ragoussis *et al*., 2006). It relies on direct mass determination of amplicons rather than indirect analysis by fluorescent or radioactive tagging (Tost and Gut, 2002) and is amenable to automation (Griffiths *et al*., 2000). It is compatible with 384-well amplification systems and is a homogeneous, automated system, minimizing sample handling. Currently, 3840 samples can be run at a time, and the iPLEX chemistry has been proven to be cost-efficient (around a 'few cents' per genotype) when multiplexes of 25–29 SNPs on 384 samples are processed (Ragoussis *et al*., 2006).

PCR amplification of the target still remains a bottleneck in most genotyping applications (Galvin, 2002) including MALDI-TOF-based genotyping. The high start-up cost and maintenance of instrumentation is further exacerbated by expensive, single-use chips and large numbers of oligos during assay design and validation. Thus, cost-effectiveness can only be achieved by high levels of multiplexing, which has been facilitated by recent advances in Assay Design software (Ragoussis *et al*., 2006). The potential to reduce the cost per assay lies in the sensitivity of the instrument. Only 15 nl of analyte is spotted on to the MassARRAY chip, despite 5–10 µl PCR amplification volumes; therefore, significant savings could result from developing miniaturized nanolitre-scale PCRs (Ragoussis *et al*., 2006). For more comprehensive discussion of DNA and SNP analysis by MALDI-TOF MS, see Griffin and Smith (2000), Ragoussis *et al*. (2006) and Tost and Gut (2006).

In silico SNP discovery

Many databases are publicly available online and *in silico* SNP discovery is a relatively inexpensive process. EST databases consist of single-pass sequences of cDNA libraries and are particularly attractive resources for SNP discovery. Of the 40 million ESTs currently lodged on GenBank, over 430,000 are from barley. Other useful barley EST databases include CR-EST (http://pgrc.ipk-gatersleben.de/est/index.php), HarvEST (http://harvest.ucr.edu), BarleyBase (http://www.plexdb.org/plex.php?database=Barley) and the Barley SNP database (http://bioinf.scri.ac.uk/barley_snpdb/).

While the quality of individual EST data is lower than some other sequence resources, the sheer volume and redundancy of data available makes EST databases attractive for SNP discovery. A large portion of the libraries is obtained from different individuals and assembly of overlapping sequences from the same loci in different individuals can enable the discovery of new SNPs (Picoult-Newberg *et al*., 1999). Unigenes assembled by aligning (or clustering) multiple ESTs from the same clone are more reliable than those from individual ESTs alone; however, the success of any SNP mining strategy depends on culling EST sequence errors by imposing stringent computational analysis of the data pool (Picoult-Newberg *et al*., 1999). In the past, sequence trace files were required to unambiguously filter sequencing errors; however, a number of software algorithms have become available which

automatically call bases depending on the level of stringency set by the user, for example, Sputnik (Rudd *et al*., 2003) and SNiPpER (Kota *et al*., 2003). *In silico* SNP discovery has thus become a relatively simple but accurate process. Once an adequate number of SNPs have been identified and verified by re-sequencing, a sensitive, robust and inexpensive detection method is subsequently required.

The number of second generation assays listed here along with applications directly related to barley genotyping demonstrates the importance of these assays in widening our understanding of plant genomics, and their ability to generate markers useful to plant breeding. With the exception of MALDI-TOF MS, second generation assays still lack the capacity to offer HTP and ultra-HTP screening for SNPs and other markers. This is primarily due to bottlenecks caused by the reliance on PCR to generate the target allele of interest, the cost of sequencing and limitations imposed by the two-dimensional nature of assays such as microarrays.

Third Generation Assays – Nanotechnology

In the near future, HTP and ultra-HTP SNP genotyping will be facilitated by: (i) generating novel materials by microfabrication to lower cost and increase capacity; (ii) reducing reaction volumes; (iii) increasing reaction rates; (iv) automating sample handling; and finally (v) developing single-platform genotyping, data analysis and storage instrumentation (Galvin, 2002). Advances in the field of nanotechnology are generating devices which are capable of meeting these requirements, thereby providing powerful new opportunities in molecular diagnostics and HTP screening (Jain, 2005). Currently these technologies are limited to frontier clinical applications; however, they will undoubtedly make their way into plant genotyping research, fast-tracking an abundance of opportunities to the plant breeding community and plant-based agriculture in general.

Nanotechnology concerns materials or structures with at least one dimension less than 100 nanometres (nm) (Lim, 2004), i.e. approximately 1/1000th the width of a human hair. Significantly, nano-sized compounds exhibit different properties to those of the same compound at macro- or micro-scale (D'Aquino *et al*., 2006). The size-dependent properties include electrical conductivity, magnetic coercivity, mechanics (hardness, strength and ductility), luminescent efficiency, transparency, catalytic properties and reaction rates (Lim, 2004).

Nanobiotechnology is the union of engineering and molecular biology with the goal of developing structures and devices of atomic, molecular or supramolecular size (Jain, 2003). The novel properties of these nanodevices have vast biological applications in diagnostics, drug delivery and therapeutics. Indeed the number of nano-industries emerging from nanobiotechnology continues to grow rapidly particularly in the field of medicine, promising 'to consign current technologies to obsolescence' (Seetharam, 2006). Nano-therapeutics are destined to attract the greatest attention of the nano-revolution as targeted drug

delivery and non-invasive medical procedures become more widely available in the fight against diseases such as cancer (Moos and Barry, 2006; Shelley, 2006). However, some of the most recent and successful applications of nanobiotechnology are in the field of molecular diagnostics (D'Aquino *et al*., 2006; Wispelwey, 2006). Following is a short review on nanoparticle-based assays with particular promise in molecular recognition and diagnostics.

Quantum Dots and FloDots

Quantum dots (QDs) are the most popular nanoparticles used in diagnostics (Jain, 2005). QDs are semiconductor nanocrystals, the most common being made of a CdSe core, often capped with ZnS to increase quantum yield (i.e. ratio of absorbed and emitted light) (Chan *et al*., 2002). Semiconductors are formed by adding conductive metal atoms on to the surface of insulators – a process known as 'doping'. The addition of conductive metal atoms to the neutral surface of an insulator changes the availability of electrons. Upon excitation, electrons are free to move into vacant orbitals and carry a current; thus, semiconductor QDs are capable of absorbing and emitting light energy as photons.

The emission properties of QDs are size- and composition-dependent. For example, a 3 nm CdSe nanoparticle will emit green light, whereas a 6 nm CdSe nanoparticle will emit red light (Xu *et al*., 2003); hence, emission wavelengths from blue to near infrared can be excited from a single wavelength (Chan *et al*., 2002), simplifying the instrumentation required for detection. QDs are an attractive alternative to traditional fluorescent dyes for a number of reasons. The QD emission spectrum is narrow and nearly symmetric, whereas its excitation profile is broad and continuous (Chan *et al*., 2002). In other words, spectral overlap between fluorescent signals is minimized and sources of excitation light can be simple and cheap. QDs can themselves be linked to biomolecules. However, they are most useful when embedded in microspheres where their optical properties can be multiplexed. In theory, a combination of QDs emitting six different colours at six different intensities could yield around 40,000 unique optical codes (Han *et al*., 2001; Ng and Liu, 2006). Thus, large screening libraries can be created by conjugating allele-specific oligonucleotide probes to microspheres encoded with unique optical addresses via specific QD composition (Han *et al*., 2001). The optical signal of the microsphere identifies the target, while the labelled target/probe complex indicates presence or absence and abundance of the target (Han *et al*., 2001). QD-encoded microbeads can be imaged on standard microscopes; however, HTP detection and screening via regular flow cytometers is possible at the rate of 1000 beads/s (Gao and Nie, 2004). The main disadvantage to QDs is that the toxic CdSe core must be coated to render them biologically inert; however, for clinical diagnostics and treatment, there is still concern that some of the toxic ion may escape (Hogan, 2006). In addition to toxicity problems, QDs are subject to poor solubility, low quantum yield and agglutination problems (Yao *et al*., 2006).

A more recent development in fluorescent nanoparticle technology is FloDots (Yao *et al*., 2006) (developed at, and named after, the University of Florida). In comparison to the size-dependent emissions of CdSe-cored QDs, FloDots consist of a silica matrix embedded with thousands of luminescent dye molecules. Dye-doped FloDots have strong emission signals when properly excited and similar photo stability to QDs. The silica surface can be modified to contain functional groups, used as a substrate for immobilization of biomolecules, and has the advantage of promoting dispersion in water, overcoming some of the poor solubility and agglutination problems of QDs (Yao *et al*., 2006).

Microspheres and Bead-based Assays

Polystyrene, latex and silica microspheres have been developed as solid platforms for attaching biological recognition compounds in what can be collectively called bead-based assays. Microspheres (2–500 μm in diameter) are functionalized to allow the attachment of multiple compounds on each bead (Battersby *et al*., 2000; Trau and Battersby, 2001). The spherical nature of the beads allows spatial homogeneity of probe attachment and subsequent target hybridization (Spiro *et al*., 2000). The small bead size allows them to stay suspended in solution for several hours without remixing, while the surface area of the beads in solution promotes close proximity of probe and target, generating near-fluid-phase reaction kinetics which is faster than that of microarrays (Fulton *et al*., 1997; Spiro *et al*., 2000). Microsphere-based assays have the potential to generate very large combinatorial libraries ($>10^{10}$ compounds) (Battersby *et al*., 2001) by varying the number and intensity of covalently attached dyes. For example, a library of 4^{16} probes can theoretically be encoded with just six fluorophores (Battersby *et al*., 2001).

Luminex

The Luminex analysis system (formerly known as FlowMetrix) is an early example of fluorescent microsphere technology. FlowMetrix polystyrene beads were impregnated with red and orange fluorophores in different ratios to produce a library of up to 100 bead types, distinguishable by relative intensities of red and orange fluorescence. Up to 2×10^6 capture probes complementary to the target of interest can be attached to the uniquely encoded microsphere (Fulton *et al*., 1997). Fluorescent wavelength and intensity were detected and analysed using computer-enhanced flow cytometry. Specific PCR targets were identified by the red-orange microsphere colour code and quantified by their green fluorescent label. Smith *et al*. (1998) used FlowMetrix for simultaneous detection of both RNA and DNA viruses. The assay was rapid, sensitive, and cross-hybridization was not observed except in the presence of extremely large amounts of PCR product ($>10^{10}$ copies). Spiro *et al*. (2000) hybridized nucleic acid sequences to the surface of polystyrene beads

(Luminex, Texas) to identify and quantify prokaryotic DNA sequences in heterogeneous environmental samples. The bead-based assay indicated superior specificity compared with microarrays and allowed accurate quantification of the target (Spiro *et al*., 2000). These assays were subsequently multiplexed successfully (Spiro and Lowe, 2002).

In a variation on the theme, the properties of complementary base pairing were used to alter the fluorescence of encoded polystyrene microspheres by FRET (Ihara *et al*., 2004). Target DNA was complementary to oligos covalently immobilized to the surface of a pair of different coloured microspheres. Thus, when hybridized to multiple targets, the microspheres aggregate, producing a FRET-induced change to particle fluorescence, measurable by fluorescent microscopy (Ihara *et al*., 2004). This technique requires some modification, however, for HTP applications and improvement in colour differentiation (Ihara *et al*., 2004).

Qbeads

Qbeads (Xu *et al*., 2003) are latex microspheres embedded with QDs. In some ways similar to the Luminex assay, Qbeads are colour-coded with QDs of varying colours and intensity; however, the assays differ primarily in the dyes used and the allele-specific target hybridization method. Optically encoded Qbeads are conjugated with allele-specific oligos and subsequently hybridized to biotinylated PCR targets. A further step is required to hybridize a streptavidin-PE-Cy5 label to the PCR target, followed by flow cytometric analysis and decoding (Xu *et al*., 2003). When validated by comparison with TaqMan PCR, the Qbead assay called homozygous and heterozygous SNP alleles with 100% accuracy compared with sequencing, which was 96.5% accurate, possibly due to the susceptibility of sequencing to poor template quality, even after PCR template clean-up with a commercial kit (Xu *et al*., 2003). As little as 0.2 ng of genomic DNA per ten SNP genotype calls was used, thereby saving genomic template, and given that only one multiplexed amplification step was performed, time and expense associated with PCR and commercial column purification products were reduced (Xu *et al*., 2003).

Silica microspheres

Silica microspheres are an attractive alternative to polystyrene beads as they are more stable in most solvent environments (Corrie *et al*., 2006). Silica microspheres are porous (Johnston *et al*., 2005), facilitate biomolecule attachment via a range of surface reactions (Corrie *et al*., 2006) and can be optically encoded by covalent attachment of fluorescent dyes (Johnston *et al*., 2005). The dyes may be incorporated in a combinatorial 'split and mix' manner, producing a diverse range of optical signals (Johnston *et al*., 2005). Oligonucleotides coupled to the surface of the microspheres fluoresce brightly upon hybridization to a perfectly matched, labelled target, whereas mismatched probes show significantly

less fluorescence (Johnston *et al*., 2005). Further refinement led to the development of thiol-functionalized organosilica microspheres with a uniformly narrow size distribution (Miller *et al*., 2005). The microsphere surface was functionalized with either thiol or amines and it was found that dye molecules covalently bound to free thiol groups were distributed uniformly throughout the microsphere interior, whereas amine functionalized microspheres became more impermeable, resulting in the dyes localized to the surface (Miller *et al*., 2005). The organosilica microsphere retained fluorescent intensity under phosphoramidite DNA synthesis conditions, whereas commercially available polystyrene-divinylbenzene microspheres with non-covalently bound dyes lost significant fluorescence (Miller *et al*., 2005). Fluorescence of an A/T mismatch was 20% less than that of a perfectly matched target (Miller *et al*., 2005), which, while quantifiable by flow cytometry, may be necessary to improve probe-target specificity for SNP detection.

Fluorescent microspheres can be detected and quantified either by fluorescent microscopy or flow cytometry. Flow cytometers were originally designed to automate optical and fluorescent measurement of cells or particles in solution and fluorescent microspheres have played an integral role in flow cytometry as calibration standards (Wedemeyer and Potter, 2001). Flow cytometry is capable of analysing multiple wavelengths of fluorescent light from thousands of particles per second; thus, when coupled to molecules of interest, hundreds of thousands of bead-based assays can be examined very quickly (Iannone, 2001). This capacity makes flow cytometry an attractive instrument for HTP detection and quantification of a wide range of molecular interactions (Nolan and Sklar, 1998).

Gold Nanoparticles

Gold nanoparticles are attractive labels for biosensors because a range of analytical techniques can be used to detect them, including fluorescence, optical absorption and electrical conductivity (Jain, 2003). Storhoff *et al*. (2004) used gold nanoparticle probes to detect unamplified bacterial DNA. When hybridized to the target and spotted on to a glass slide, gold-labelled oligonucleotide probes (GNP-DNA) scattered yellow-orange light when illuminated with white light from the side. Unhybridized probes scattered green light. Visual readout removes the need for complex detection instrumentation while the assay itself is rapid and inexpensive, without the need for a PCR amplification step (Storhoff *et al*., 2004).

In a different approach, GNP-DNA was used to discriminate SNPs in genes associated with thrombotic disorders, from unamplified human genomic DNA (Bao *et al*., 2005). Briefly, a capture probe specific to the allele of interest and a gene-specific signal probe were designed to 'sandwich' the target DNA. Capture probes were hybridized to a microarray and signal probes were modified with a gold nanoparticle (GNP-DNA). Genomic DNA, fragmented by ultrasonication into 500 kb lengths, was then hybridized to the microarray-bound allele-specific capture probes under stringent conditions.

Non-specific hybridizations were removed by washing, and a second hybridization step bound the GNP-DNA to the genomic DNA target, thus sandwiching the target between the capture and signal probes. Signal strength was enhanced by precipitating elemental silver on to the nanoparticles. The silver-amplified gold nanoparticles were excited with white light and the scattered light was captured on a photo sensor. While allele-specific identification required two hybridization steps and silver precipitation to enhance the signal, the authors were able to reliably genotype SNPs from 150,000 genome copies or 500 ng human genomic DNA in approximately 1 h.

Nanobarcodes

Nanobarcodes (Nicewarner-Pena *et al*., 2001) are microscopic metallic nanowires (Sha *et al*., 2006) which are 'barcoded' by sequentially electroplating metal ions of different reflectivity into narrow channels using a lithographic process. Complex barcodes are achieved by varying sequence and type of metal being deposited. Analytes bound to the particle by affinity-capture are detected by fluorescence, and the differential reflectivity of the barcode enables identification by conventional light microscopy (Nicewarner-Pena *et al*., 2001). Sha *et al*. (2006) were able to discriminate SNPs within the cytochrome P450 family of genes using nanobarcodes. Each uniquely coded nanowire is fixed to a different oligonucleotide probe and added to the reaction along with PCR target and fluorescently labelled probes. In the presence of a perfect match, the probes and PCR target hybridize and the probes are enzymatically ligated. The nanowires are imaged and analysed to determine which allele is present.

Nanotechnology on a Chip – NanoChips and NanoArrays

The BioForce Nano eNabler, formerly known as the NanoArray (BioForce Nanosciences, USA), is the next generation, ultra-miniaturized version of the microarray, capable of ultramicro- and nanoscale fluid delivery. Thousands of 1–20 μm spots of nucleic acids, antibodies and nanomaterials such as QDs, colloids and other material can be direct-patterned by the Nano eNabler via a micro-cantilever print head. Up to 400 nanospots can now be arrayed in the same area as the traditional microarray spot, vastly increasing the number of samples which can be analysed (Jain, 2003).

Conclusions

As experienced in the silicon revolution, miniaturization is the key to reducing plant genotyping costs and increasing throughput. Rapid advances in SNP discovery are providing vast amounts of data, ready for application in detection and diagnostics, pharmacogenetics and epidemiology. The priority

now is developing cost-effective platforms capable of rapidly and accurately identifying genetic polymorphisms (Shi, 2001) combined with multiplex and automated HTP detection capabilities. The challenge for HTP plant genotyping lies in continued SNP discovery, 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 perhaps the affordability of testing and validating novel HTP genotyping assays. First and second generation genotyping methods discussed here have contributed enormously to the wealth of data available and to our current capacity for generating and detecting molecular markers; however, apart from MALDI-TOF MS, cost-effective, HTP, multiplex genotyping is still largely unavailable.

In future, plant genotyping, elimination of PCR amplification is desirable as, in addition to the extra steps required to generate target amplicons, cross-contamination and variability in target amplification efficiency are inherent flaws to PCR in HTP applications (Griffin and Smith, 2000). In order to remove the requirement for PCR target enrichment, detection sensitivity will become the priority (Galvin, 2002) particularly in large genomes such as barley, where the number of repetitive elements may weaken the allele-specific signal (Rostoks *et al*., 2005a). At present, gold nanoparticle assays are sensitive enough to detect non-amplified targets (Storhoff *et al*., 2004; Bao *et al*., 2005); however, they are array-based. Multiple washings, hybridizations and silver precipitation to enhance signal increase time and labour required and reduce HTP potential.

Bead-based assays have the advantage of near-fluid-phase reaction kinetics which are rapid compared with the relatively slower kinetics of microarray-based technologies (Xu *et al*., 2003). Conjugated to specific oligo recognition events, bead-based assays have the potential to realize HTP SNP genotyping due to the low cost, small reaction volumes, rapid near-fluid-phase reaction rates and amenity to automated sample handling. This combined with the single-platform genotyping, data analysis and storage available in flow cytometry may provide the plant sciences with the most powerful tool to date in the quest for cost-effective genotyping.

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