Plant Breeding
doi:10.1111/j.1439-0523.2007.01306.x

Automation of DNA marker analysis for molecular breeding in crops: practical experience of a plant breeding company

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With 2 figures and 2 tables

Received March 10, 2006/Accepted June 17, 2006
Communicated by F. Ordon

Abstract

In modern plant breeding, DNA marker analyses are of increasing importance and, as the methods become more widely adopted, the capacity for high-throughput analyses at low cost is crucial for its practical use. Automation of the analysis processes is a way to meet these requirements. In order to achieve this, while keeping adequate flexibility in the analysis processes, Svalöf Weibull AB (SW) has developed a fully automated polymerase chain reaction system. It has been evaluated on barley and canola lines and is capable of analysing up to 2200 samples per day at a cost of 0,24 € per analysis for marker-assisted selection and quality control of genetically modified organisms. A detailed description of this system is given, and improvements to the throughput and applications are discussed.

Key words: DNA marker analysis — marker-assisted selection — genome characterization — automation — practical plant breeding — PCR

Over the past decades, molecular marker technology has evolved into a valuable tool for plant breeding. A number of techniques [e.g. restriction fragment length polymorphism, amplified fragment length polymorphism, simple sequence repeats (SSR), single nucleotide polymorphisms] can be used as DNA markers linked to traits of interest, directing selection towards these markers instead of a phenotypic reaction (Edwards and Mogg 2001). In addition, fingerprinting of breeding lines using molecular markers, as well as detailed genome analysis of plants, together provide an important tool to characterize and protect germplasm (Lombard et al. 2000). Quality assurance of, for example, genetically modified organisms (GMOs) and hybrids is another important molecular marker application for use by plant breeding companies.

Traditionally, new cultivars have been developed by phenotypic selection of superior plants (Edwards and Mogg 2001). Today, selection of desirable genotypes can also be done directly at the DNA level, all year round and regardless of developmental stages and of field/greenhouse trials. As several markers can be combined, new possibilities to incorporate different genes into the same line are available to the plant breeders (Hospital 2003) and superior early generation segregants may be selected by using molecular markers for key genes (Werner et al. 2005). Furthermore, DNA marker techniques are non-destructive, and heterozygous individuals may be identified and maintained more easily than by conventional means (Helentjaris 1991). At SW, more than 50 trait-linked markers are used by plant breeders to complement phenotypic selection.

Molecular Marker Technology in Practical Plant Breeding

Molecular marker tools, now available in plant breeding, can considerably improve important breeding steps.

First, marker-assisted selection (MAS) can both improve selection and the efficiency of field trials by replacing complex and sensitive selection tests, e.g. barley yellow mosaic virus (Tuvesson et al. 1998), phoma in *Brassica* (Foisset et al. 1998) and by discarding undesirable genotypes earlier, thus reserving field trials for more appropriate individuals for the evaluation of complex traits in later generations. MAS performed in early generations (Liu et al. 2004), though providing more accurate results, also dramatically increases the frequencies of superior genotypes. Models show that a breeder using MAS needs to test 1.0–16.7 times less progeny than a breeder using phenotypic selection so as to be assured of selecting one or more superior genotypes (Knapp 1998). This underlines the necessity, from a breeding perspective, of a methodology which allows plant material to be monitored using realistically high numbers of individuals in the early generations.

Secondly, several breeding steps can be speeded up, e.g. multiple selections, homozygosity or uniformity checks, backcrosses, etc. It has been demonstrated that the proportion of a recurrent parent in the first generation of marker-assisted backcrosses (MAB), as described by Tanksley and Rick (1980) in their model, corresponds to that expected after three generations of conventional backcrosses (Allard 1960). Considering that Allard estimated the adequate number of generations to be six, MAB would represent a considerable gain in time. However, the number of markers and material to be screened would be very large (Melchinger 1990).

The high number of individuals and the economic constraints involved in a breeding programme require that molecular markers are technically easy to use, cheap and informative (Hernandez 2004). The polymerase chain reaction (PCR) (Saiki et al. 1988) is technically a simple and quick method, requiring only small amounts of crudely-extracted DNA and its procedures are amenable to automation, which is essential in the rapid processing and analysis of large numbers of genotypes used in plant breeding programmes.

Automation of Molecular Marker Analysis

Lydiate (1999) has described efficient genomic research as a three step procedure: (1) automating what can be automated, (2) speeding up the process and (3) provision of molecular shortcuts. A similar approach to automate molecular marker analyses has been used at SW. However, the automation of PCR procedures introduces a few problems. First, amongst the PCR-based markers, there is no single established or universal marker technology, and each type of marker might require its own procedure. Marker technology as a whole is still in a growing phase and evolving rapidly, so that technologies, as well as the availability of appropriate markers, may lead to delays in exploiting marker applications. Finally, with DNA-marking being a broad concept, each specific application might require its own marker technology or present different technical challenges.

The SW Experience in Automating DNA Marker Analysis

When looking, in a practical plant breeding way, at the relationship between the number of markers and the number of individuals, DNA marker projects can be divided into two main groups as seen in Fig. 1.

It was decided first to limit the choice of marker technologies by trying to focus exclusively on PCR-markers. For example, microsatellite technology (SSR) is one type of marker that fulfils most of the requirements necessary in practical plant breeding. They are PCR based, easy to use and amenable to automation. They are well spread on the genome and are generally highly informative. Furthermore they are widely available and well described in most crops. Finally they are applicable in the two main groups described above and in Fig. 1 (Werner et al. 2005, Kolodinska-Brantestam et al. 2006).

The processes were then standardized by working solely on a microtitre plate format from start to finish. For both applications a well thought out and flexible system is required. A core system is preferred as, besides its flexibility, it increases the reliability of the system, reduces start-up time and enables different assays to be run simultaneously (Brandt 1998). SW developed, in collaboration with Thermo CRS (Burlington, Canada; http://www.crsrobotics.com), a fully automated system with the main emphasis being on flexibility and high throughput capable of producing more than 4000 PCR reactions per day or visualizing up to 2200 PCR reactions per day, which is the limiting factor. The system includes different peripherals that are served by a robotic arm, as described in Fig 2. These machines have been chosen not only for their individual automated performance but also because they all feature an open architecture that allows their nests to be accessible by the robotic arm, thus enabling full automation. Thermo CRS of Canada have supervised the integration of these peripherals into one system. Their software *Polara*™ enables an easy set-up and scheduling of the procedures and controls the peripherals, either directly or through their own software, hence freeing the procedures from any human intervention. As it is often the major cost, a decrease in hands-on will also decrease the cost of the analyses (Klapper et al. 1998).

It is estimated that both groups can be accomplished using a common core set-up. A set-up has been designed (Fig 2), described as follows: the robot arm picks up DNA plates, stored at +5°C in the carousel, and places them on the pipetting robot. The appropriate PCR mix is dispensed into the PCR plates, the reaction volume varies between 5 and 25 µl. A volume of 1.5 µl DNA is then transferred to each of the PCR wells. Between each transfer the tips of the pipetting robot are thoroughly washed to avoid cross-contamination. The DNA plates are moved back into the carousel while the PCR plates are sealed to avoid evaporation. The PCR plates are then placed in the thermocycler and the appropriate PCR is performed, after which the PCR plates are placed in the carousel while waiting for visualization. Prior to visualization the PCR plates are pierced, the samples are then either directly

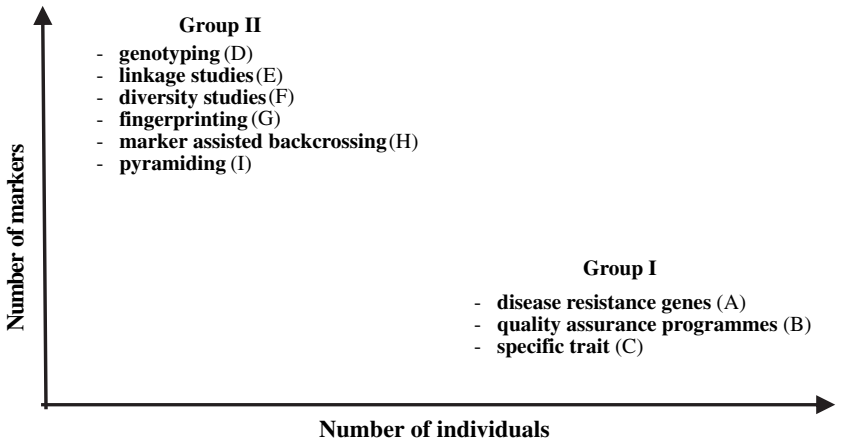

Fig. 1: Division of DNA marker projects depending on the relationship between the number of markers/number of individuals. The ranking within groups has been made arbitrarily and may not be representative, as the figures vary between crops and studies: (a) e.g. phoma in Brassica (Foisset et al. 1998); barley yellow mosaic virus (Tuvesson et al. 1998); (b) e.g. hybridity control; adventitious presence of genetically modified sequences (Delano et al. 2003); (c) e.g. male sterility in Brassica (Primard-Brisset et al. 2005) using internal markers and expression of final attenuation in malting (Frank Rath, personal communication); (d) to cover the entire genome e.g. association mapping (Gant and Long 2003); (e) to locate and link molecular markers to a trait of interest (Ivandic et al. 1998); (f) to assess genetic diversity in crops (Kolodinska-Brantestam et al. 2004); (g) to characterize varieties (Lombard et al. 2000); (h) to use a representative set of markers in order to efficiently select the recipient's genetic background in the offspring when crossing with exotic relatives (Åhman et al. 2000); and (i) to use a set of markers, each specific to e.g. disease resistance genes, in order to combine them in the same genotype (Werner et al. 2005)

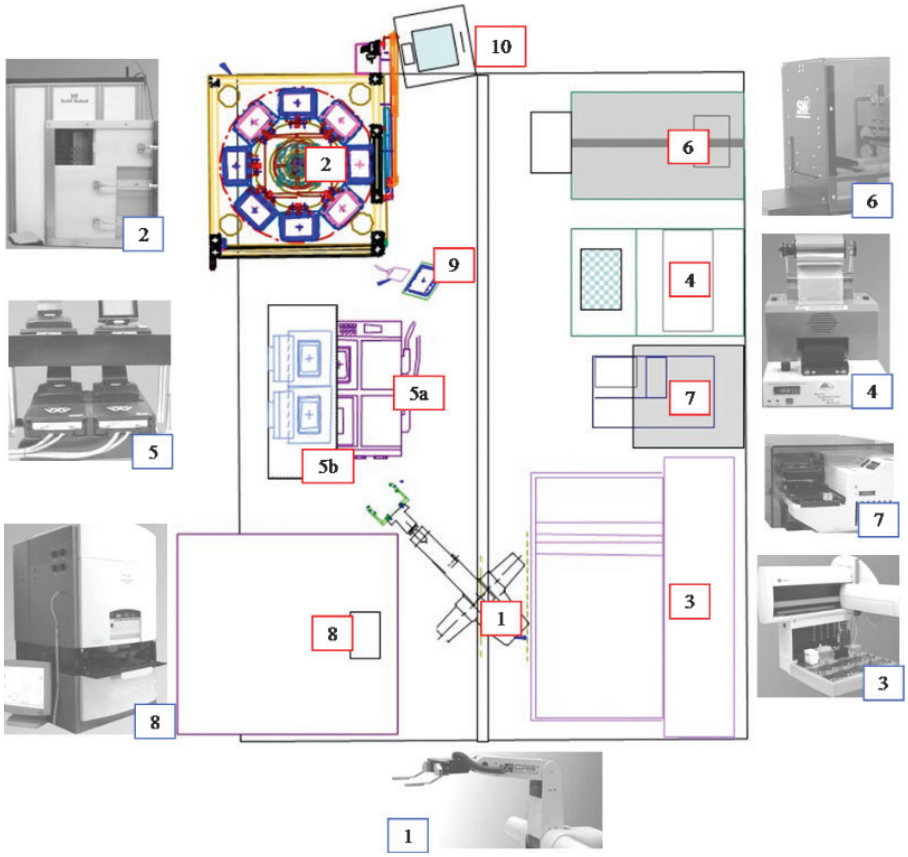

Fig. 2: Illustrated blueprint of the fully automated PCR amplification system

1	*T265* robotic arm mounted on a 2-m rail track	Thermo CRS, Canada	Transporting and serving plates to the other peripherals.
2	Refrigerated carousel	Thermo CRS, Canada	Housing 120 nests (1 per plate) for cold storage.
3	*Multiprobe II*	Packard, USA	Automated pipetting robot with eight accessible nests for sensitive liquid handling.
4	*APLS100*	AB Gene, UK	Sealing of PCR plates.
5a	*Tetrad PTC-225*	MJ Research, USA	PCR thermocycler with four independent PCR-blocks with automated lids.
5b	*Remote Alpha Dock Connectors*	MJ Research, USA	Satellites for two blocks placed on a shelf.
6	Piercer	Thermo CRS, Canada	Unsealing the PCR plates after amplification.
7	*Multidrop 384*	Thermo Labsystems, Finland	Automated dispensing robot for rapid mix dispensing.
8	SC*E 9610*	Spectrumedix LLC, USA	Automated 96 capillary electrophoresis apparatus with 6 accessible nests.
9	Barcode reader	Thermo CRS, Canada	Registration of plate barcode.
10	Air-tight waste	SW, Sweden	Disposal of plates containing formamide

visualized using 96-capillary electrophoresis or first denatured with formamide containing an internal line standard. The whole process is performed in a fully automated manner.

Materials and Methods

Two different examples have been chosen to illustrate the different application types.

Many genotypes – few markers: Non-fluorescently dyed amplicons are directly visualized replacing agarose-gel based analyses of one or more trait-linked markers (group I in Fig. 1). This type study is especially applicable for MAS or for quality assurance programmes to monitor the adventitious presence of GMOs in commercial varieties.

Hordeum vulgare and *Brassica napus* lines originating from SW breeding programmes were used. Using a paper-punch, leaf cuts of 20 mm^2 were taken from individual plants and placed in a 96-format plate. Two people can harvest up to 3840 leaf samples per day. When handling GM material, special precautions, such as sterilizing the punches to avoid possible cross-contamination, must be taken into consideration, which will reduce the harvesting capacity by one quarter. The DNA isolation is then performed according to a quick DNA extraction protocol (Dayteg et al. 1998) enabling the DNA from 96 samples to be extracted within 10 min (theoretically more than 4000 in a working day). When handling barley seed samples, a non-destructive seed-based DNA extraction protocol is used, as described by von Post et al. (2003), to extract approximately 700 seeds per day.

Either a set of GM-specific markers, chosen to monitor the adventitious presence of any GM registered worldwide (http://www.agbios.com) or markers linked to specific traits of interest is used. These either originate from the literature, e.g. barley yellow dwarf virus resistance *Yd2* (Paltridge et al. 1998), leaf rust resistance *Rph7* (Mano et al. 1999), they may be provided by business partners (Monsanto, St. Louis, MO; Bayer CropScience, Saint-Jean-Sur-Richelievu, Québec),

they may be privately developed (DNA LandMarks Inc., PA, Canada) or bought as malting quality markers linked to final attenuation quality (Versuchs und Lehranstalt für Brauerei, Berlin, Germany). Before large-scale use, the markers are evaluated for their diagnosticity. The PCR is performed according to the respective protocol of each molecular marker. The PCR amplicons are then directly visualised on the 96-capillary electrophoresis. Collaboration with Spectrumedix LLC (State College, PA http://www.spectrumedix.com), led to the development of a protocol for automated fragment analysis that uses ethidium bromide in the capillaries' gel matrix instead of using fluorescent dyes, thus reducing the chemical cost. Software has also been developed (IDGene^{TM}) that automatically scores the presence or absence of the expected fragment, making the analysis of the data more efficient.

Many markers – few genotypes: Fluorescently-dyed amplicons are directly visualized, which can advantageously replace polyacrylamide gel-based electrophoresis (group II in Fig. 1). This type of study is especially applicable for whole genome studies, e.g. MAB, genetic relationship studies, etc.

Kolodinska-Brantestam et al. (2006) performed a diversity study on spring barley using SSR markers. The study was performed manually but established protocols and a data core set that enable a smooth and reliable transfer to an automated method.

A total of 852 individual plants from 197 spring barley (*Hordeum vulgare* L. ssp. *vulgare*) accessions were studied. The lines originate from the different Baltic and Nordic countries and cover several cultivation periods (from landraces to modern elite varieties). DNA was extracted using a Quick standard method adapted from Cheung et al. (1993). Three SSR markers per chromosome were chosen from the SSR set developed by Macauley et al. (2001). Twenty-one markers were considered sufficiently informative because they all have high PIC (Polymorphism information content) values (average of 0.71) and are well distributed throughout the barley genome. The PCR reaction was carried out in a total volume of 5 µl and performed according to the programmes from the Scottish Crop Research Institute for each primer pair (Macauley et al. 2001). The samples were denatured in a post-PCR step by simply diluting a fraction of the PCR product in a solution containing formamide and an internal lane standard (ILS). The samples were then analysed on the 96-capillary sequencer (Spectrumedix LCC, USA) and the electropherograms processed by the DNA fragment analysis software Genospectrum^{TM} version 2.06.

Results

With the prospect of automating DNA marker processes, the focus was on increasing the throughput and lowering the cost for the two groups and specifying two specific sets of requirement as highlighted in Table 1.

In group I (MAS), this was achieved by decreasing the procedure time. More individuals have to be processed in very short time. As few markers will be screened, neither DNA quality nor pipetting accuracy will be a major issue, and therefore simplified, quick and reliable processing are important. Because the assays are well described, the visualization of the results can be focused and limited to the expected fragments for more efficient data acquisition. In group II (genotyping), this will be achieved by increasing the amount of data per individual. One way of achieving this is by multiplexing but this sets higher requirements on DNA quality, automated liquid handling and data analysis (Mace et al. 2003). In both cases, the capacity is further increased by the possibility of an extended assay capacity (overnight and weekend runs). At SW, ca 200 000 data points are analysed annually (dp, total number of marker alleles scored across all samples), two-thirds belong to group I (MAS/QAP) and one-third to group II (genotyping).

Table 1: Set of requirements for the automation of DNA markers. + lower importance + + higher importance

Requirements	Group I: many individuals/ few markers	Group II: few individuals/ many markers
Quality		
DNA	+	+ +
Pipetting	+	+ +
Visualization	+	+ +
Speed		
DNA	+ +	+
Pipetting	+ +	+
Visualization	+ +	+
High throughput through	Speed	Multiplex

Many genotypes – few markers

The ability to use ethidium bromide for visualization enables one not only to speed up both the data acquisition and analysis time but also to cut down efficiently on the analysis cost (group I in Fig. 1). Comparisons were made with a similar assay visualized with an agarose gel-based electrophoresis (Table 2). Though the investment cost is much higher (expressed as yearly depreciation) and the analysis cost is higher (i.e. capillaries, gel matrix), the assay cost per sample using the automated system decreased from 0.30 to 0.24€. This was achieved by the important decrease in reaction volume (down to one-fifth) associated with the equipment sensitivity. Furthermore, the hands-on time per plate has also been substantially reduced, hence further reducing the total sample cost while increasing the overall productivity.

Table 2: Comparison of the DNA fragment visualization methods

	Agarose electrophoresis	Capillary electrophoresis ethidium bromide	Capillary electrophoresis fluorescent dyes
Extraction cost	1.5€	1.5€	82€
PCR cost	20.5€	6.2€	8.3€
Analysis cost	5.6€	6€	18.2[2]€/10.3[3]€
Depreciation[1]	0.9€	9.3€	9.3€
Hands-on time (average)	55 min	16 min	50 min
Throughput (per day)	900 samples	2200 samples	900 samples

The values are calculated per plate (96 samples) at their respective capacity. The capillary electrophoreses have been performed on the SCE9610 (Spectrumedix LCC). All figures are given for a simple reaction (simplex). The extraction cost includes the material and consumable costs. The first two columns are for the quick extraction protocol (4000 sample capacity) while the last is for the automated standard protocol (800 sample capacity). The PCR cost includes the material and consumable costs for the respective applications. The last two columns do not include the cost of tips as they are performed on the robot. Furthermore, their PCR volumes have been decreased to one-fifth and one-third, respectively. The analysis cost includes the material (e.g. capillaries) and consumable costs. There are two figures for the last column, as the use of commercial or home-made ILS strongly influences the cost. Labour is calculated as the average time. Differences in the depreciation cost are due to the cost of the equipment.

[1]On a 10-year basis and 200 000 analyses per year.

[2]Use of commercial ILS.

[3]Use of non-commercial ILS.

Some steps may still be improved. The use of the dispenser, when considering a low number of markers (few PCR mastermixes), would further speed up the process in a significant manner. Though not an issue for these activities, it has been pointed out that visualization, being reduced in capacity, is the limiting factor. One could probably gain in visualization capacity by multiplexing. Two aspects have to be considered at this point (1) if using ethidium bromide, the fragments should clearly be of different sizes as the results are in monochrome and (2) if using fluorescent dye, the capacity gain should justify the increase in chemical cost.

Many markers – few genotypes

The 21 primers pairs assayed 22 mapped loci with a total of 191 alleles. Kolodinska-Brantestam gathered a total of 18 744 data points (group II in Fig. 1). This data set, obtained from such a wide background, provides a reliable reference set which will facilitate a secure transfer to automation.

This study enabled protocols to be established and adapted bearing in mind their future application in a fully automated system (e.g. genetic relationship studies). The DNA extraction method allows one to extract in microtitre plate format the DNA from ca 800 leaf samples/day/person, compared to 50 manually. This has significantly speeded up the process, though still yielding a high quantity (100 µl with an average concentration of 140 ng/µl) of DNA of high quality (average $OD_{260/280}$ of 2.0). As seen in the last column of Table 2, the PCR cost is slightly higher than with ethidium bromide-stained capillary electrophoresis. This is due to a larger PCR volume and the use of fluorescent dyes. The analysis cost is, however, much higher due to more expensive gel matrix and ILS. It should also be noted that the hands-on time is greatly increased, mainly because of a more time-consuming DNA extraction protocol and data analysis. However, it is believed that a further reduction in reaction volume might be possible and that the use of multiplex reactions, though slightly increasing the PCR cost, will multiply the throughput. This would mean that from a current capacity of ca 900 dp/day at an assay cost of 1.15€/sample, the use of four multiplexed markers with an automated procedure, would increase this figure to more than 3600 dp/day for nearly the same overall sample price.

Future perspectives

Internal evaluations of the system suggest that the overall sensitivity and specificity of the automated methods are equivalent to those of manual methods. Cross contaminations among assays have not been observed (using blank controls) and variation between assays using the system is low. Moreover, the system decreases the labour and skill requirements of PCR, thus liberating qualified technical staff from tedious routine tasks. The overall impression is that with an improvement in which throughput, the use of the sequencer (SCE9610; Spectrumedix LLC) which provides greater sensitivity which in turn, as less amplicon copies are required for detection, might enable the use of even smaller reaction volumes, thus decreasing both the cost and time of the analysis. However, a review of the set-up could well be required, in order to provide optimal use of the different machines. An improvement in workflow might also include the use of a 384-well format in some steps, not only to solve future bottleneck issues but also to increase the overall throughput if required. The flexibility of the system encourages constant adjustment to provide the modifications needed in order to cope with the challenges that arise.

Acknowledgements

FORMAS and ØFORSK are acknowledged for their financial support.

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