Theor Appl Genet (2006) 112: 1115–1124
DOI 10.1007/s00122-006-0213-6

ORIGINAL PAPER

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Utilization of the three high-throughput SNP genotyping methods, the GOOD assay, Amplifluor and TaqMan, in diploid and polyploid plants

Received: 9 June 2005 / Accepted: 4 January 2006 / Published online: 2 February 2006
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Abstract The application of high-throughput SNP genotyping is a great challenge for many research projects in the plant genetics domain. The GOOD assay for mass spectrometry, Amplifluor® and TaqMan® are three methods that rely on different principles for allele discrimination and detection, specifically, primer extension, allele-specific PCR and hybridization, respectively. First, with the goal of assessing allele frequencies by means of SNP genotyping, we compared these methods on a set of three SNPs present in the herbicide resistance genes *CSR*, *AXR1* and *IXR1* of *Arabidopsis thaliana*. In this comparison, we obtained the best results with TaqMan® based on PCR specificity, flexibility in primer design and success rate. We also used mass spectrometry for genotyping polyploid species. Finally, a combination of the three methods was used for medium- to high-throughput genotyping in a number of different plant species. Here, we show that all three genotyping technologies are successful in discriminating alleles in various plant species and discuss the factors that must be considered in assessing which method to use for a given application.

Electronic Supplementary Material Supplementary material is available for this article at http://dx.doi.org/10.1007/s00122-006-0213-6 and is accessible for authorized users.

Communicated by A. Charcosset

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Introduction

The utility of SNPs in answering a large range of biological questions in a variety of fields is now beyond question. Correspondingly, a large number of methods for SNP genotyping exist: these include, but are not limited to, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), and a number of techniques that rely on fluorescence, for example sequencing, Amplifluor® (Serological Corp.), TaqMan®, SnaPshot® and SNPlex® (Applied Biosystems), Illumina® (Illumina Inc.), and chip-based technologies such as Genechips (Affymetrix; Gut 2004).

In plants, SNPs can be used as genetic markers: for many breeding applications, for population studies, for germplasm fingerprinting, for gene mapping, for genotype/phenotype association, and for positional-cloning studies. Plants offer unique challenges for genotyping studies in that the majority of cultivated species are polyploid and have extremely large genomes.

In plant species, maize is considered highly polymorphic, with an average of one SNP every 104 base pairs (bp; Tenaillon et al. 2001); soybean, a self-fertilized species, presents about one SNP every 273 bp (Zhu et al. 2003); wheat has one SNP every 200 bp (Ravel et al. 2004) and rapeseed has one SNP every 600 bp (Fourmann et al. 2002). In *Arabidopsis thaliana*, an SNP occurs on average every 336 bp when 6 accessions are compared to Columbia (*Col-0*; Schmid et al. 2003), but the level of variation greatly depends on the locus considered and may be much greater, for example in promoter regions where we have found up to 1 SNP per 12.5 bp (unpublished data and McKhann et al. 2004).

We are interested in finding an optimal protocol for genotyping in plants. The scale that we require in most cases is in the order of 1–5 SNPs for 384–768 individuals, although higher throughput is sometimes required. We chose to compare three different technologies that are available at the French National Genotyping Center (CNG). The GOOD assay (Sauer et al. 2000) is an

allele-specific primer extension protocol that employs MALDI-TOF mass spectrometry. The utility of this method has been shown for genotyping SNPs in humans (Tost and Gut 2002). Amplifluor® technology (Myakishev et al. 2001) is based on competitive allele-specific PCR amplification, while the TaqMan® PCR technology (Livak 1999) relies on allele-specific hybridization. Amplifluor® and TaqMan® are simple SNP genotyping methods where all reagents are added into a reaction well, which is then sealed and subjected to thermal cycling. The mass spectrometry-based protocol requires successive additions of reagents to a sample well and the transfer of allele-specific products onto MALDI target plates for analysis.

For our comparison, we examined three different herbicide resistance genes from *A. thaliana*. The genotyping results were used to assess the evolution of resistance allele frequencies in herbicide-free populations (Roux et al. 2004, 2005; F. Roux et al., unpublished data), one indirect method to estimate fitness cost associated with pesticide resistance (Bourguet et al. 2004). We also present the results of genotyping of other diploid and polyploid plant genomes using one of these methods.

Materials and methods

Plant material

For *A. thaliana*, a segregating population of 384 individuals issued from the selfing of a heterozygous plant at the *CSR*, *IXR1* and *AXR1* genes was used (F. Roux, unpublished data). One SNP that determines herbicide resistance (Haughn et al. 1988; Leyser et al. 1993; Scheible et al. 2001) from each gene was analyzed. The SNPs are named *csr1-1*, *axr1-3* and *ixr1-2* for the *CSR*, *IXR1* and *AXR1* genes, respectively (Roux et al. 2004). For each SNP, sixteen DNA samples were added as positive controls (12 homozygous and 4 heterozygous). DNA was extracted from 10-day-old germinated seedlings with a very simple and quick protocol (Saini et al. 1999). Before the PCR reaction, the samples were diluted 1/50.

The rapeseed plants (*Brassica napus*) were recombinant inbred lines from a cross between two inbred lines, Stellar and Drakkar. Four DNAs from each parent were added as positive controls. DNA was prepared from 380 adult plants with the cetyl methylammonium bromide (CTAB) protocol (Doyle and Doyle 1990). A pair of gene-specific primers was designed from the alignments of ADH-homologous gene sequences (Brunel et al. 1999). Other plants mentioned were all extracted using the CTAB protocol (Doyle and Doyle 1990).

DNA quantification

One microliter of 28 different *A. thaliana* DNAs were quantified using the PicoGreen dsDNA quantification kit (Invitrogen France, Cergy Pontoise) with a SpectraMAX Gemini spectrofluorometer (Molecular Devices, St. Grégoire, France). Standards were five different dilutions of calf thymus DNA (0–50 ng/μl).

Mass spectrometric analysis

The GOOD assay reaction was performed as described by Sauer et al. (2000). For each set of 384 samples, two plates are prepared and genotyped, a "normal" and a "reorganized". Briefly, the samples at the exterior of the normal plate are moved to the center of the reorganized plate. This is to avoid potential problems resulting from the position of the sample in the plate (edge effect). The details of the PCR are described in the Supplementary Materials.

Primer extension reaction

Primers for the extension reaction were determined using the OLIGO v. 6.0 program (Rychlik et al. 1990). The protocol was as described by Sauer et al. (2000) except we used 0.4U THERMIpol DNA polymerase (Solis Biodyne, Tartu, Estonia).

An initial denaturing step of 3 min at 95°C was used followed by 40 cycles of (20 s at 95°C, 1 min at 58°C and 1 min at 62°C).

The gene targets from the different species, sequence of the extension primers, SNP and expected mass are described in Supplementary Material Table 1.

Computer analysis

The Autoflex MALDI mass spectrometer for SNP genotyping provides the results from each plate with "Genotools SNP MANAGER" software (Pusch et al. 2001), which gives a summary of the results in a table that relates the well position to genotype. Quality control and genotyping analysis were performed using "SNP Master" software (program developed for internal use at the Centre National de Genotypage, Evry, France). The results were determined and a Microsoft Excel file with the consensus or individual genotypes was exported to the database.

Amplifluor® analysis

The allele-specific PCR primers and the common reverse primers (COM) were designed from each gene sequence for the different species using Amplifluor® AssayArchitect software (http://www.assayarchitect.com/, Chemicon International, a division of Serologicals Corporation, Norcross, GA, USA). Oligonucleotides were synthesized and HPLC purified by MWG Biotech (Ebersberg, Germany). One unique 21-nucleotide tail that is complementary to the Universal Amplifluor® primer (Bengra et al. 2002) was added to the 5′ end of

each allele-specific primer (Myakishev et al. 2001). Fluorescent measurements and data analysis for Amplifluor® were as described by Bengra et al. (2002). PCR conditions are described in the Supplementary Materials. The gene targets from the different species, the sequence of the PCR primers (two allele-specific primers, and a COM reverse primer per SNP) used to amplify each of the three SNPs from the *CSR*, *AXR1* and *IXR1* genes, and the length of the amplified target DNA are shown in Supplementary Material Table 2.

Computer analysis

Fluorescence results obtained on an ABI Prism 7900HT were transferred to a Microsoft Excel file for analysis, and scatter plots for each SNP locus were obtained using the SDS Software Workspace (Applied Biosystems).

TaqMan® analysis

TaqMan® (Applied Biosystems, Foster City, CA, USA) was performed according to the manufacturer's instructions using FAM and VIC reporter dyes. TaqMan® probes and primers were developed by Applied Biosystems by design (AbD). PCR conditions are described in the Supplementary Materials. Gene targets for the different species, sequences of gene-specific primers, SNPs, probe sequences, corresponding fluorochromes (dyes) and the lengths of amplified target DNA in base pairs are shown in Supplementary Material Table 3.

Fluorescent measurements, data analysis and computer analysis for the TaqMan® assay were performed using the same methods as described earlier for the Amplifluor® technology.

Results

Comparison of accuracy and repeatability of mass spectrometry (GOOD Assay), Amplifluor® and TaqMan® methods

For the direct comparison of the three methods, the genes *csr1*-1 and *axr1-3* were used since Amplifluor® failed to give results for the *IXR1* gene. For the three SNPs studied by the three different methods, the correct genotypes were confirmed using 16 previously sequenced DNA controls. In every case, each 384-well plate was genotyped twice to determine the repeatability of the technology. For each method, we estimated the percentage of data points that were successful, discordant (different genotypes for the same DNA) or indeterminate (absence of results). The results

Table 1 Comparison of the percentage of successful data point rate of the three techniques

Technology	Mass spectrometry			Amplifluor®			TaqMan®		
Gene	Successful (%)	Indeterminate (%)	Discordant (%)	Successful (%)	Indeterminate (%)	Discordant (%)	Successful (%)	Indeterminate (%)	Discordant (%)
CSR (*Arabidopsis thaliana*)	94.3	2.6	3.1	96.1	3.9	0	97	2.5	0.5
AXR (*A. thaliana*)	82	18	0	97.4	2.6	0	100	0	0
IXR (*A. thaliana*)	94.3	5.7	0	0 (nonspecific)	–	–	99.7	0.3	0

for the three SNPs as determined by the three different methods are summarized in Table 1.

Results for mass spectrometry are shown in Fig. 1 and Table 1. An example using mass spectrometry for the *CSR* gene is shown in Fig. 1a. This SNP is a T/C polymorphism. Efficient and reliable incorporation during primer extension of the two corresponding complementary α-S-ddNTPs (A and G) was observed. The masses of the allele-specific products are 1,505.5 Da for T and 1,521.5 Da for C. In two repetitions of the 384-well microplate, 94.3% of the data points gave the same genotype for both plates, 3.1% were discordant and 2.6% were indeterminate. Results were scored discordant only when two clear results were obtained, but that were not the same. Results were considered indeterminate when neither plate gave a readable result. Indeterminate results could be produced by PCR failures due to, for example, extreme DNA quantity. Results were obtained for 100% of the samples using one of the three methods, thus clearly indicating that discordant and indeterminate results did not arise due to a degradation of the DNA or other sample-related problems.

The results for Amplifluor® technology for the same polymorphism are shown in Fig. 2a and Table 1. The homozygous T genotypes (VIC fluorescence) and the homozygous C genotypes (FAM fluorescence) accumulate along the *x* or *y*-axes, respectively. The heterozygous C/T genotypes cluster along a diagonal line between the *x* and *y*-axes. The negative controls appear near the origin with virtually no fluorescence, indicating that the chemistry does not yield a result in the absence of template. 96.1% of the data points were successful.

Using Amplifluor® technology, the data points were found to be spread along the axes. We hypothesized that this is probably related to the variation in the DNA quantity among individuals. We therefore determined the DNA concentration from 28 samples of the 384-well plate used for this study (see Materials and methods). The DNA concentrations were between 0.1 and 1 ng/µl. We found that the lowest fluorescence (including no

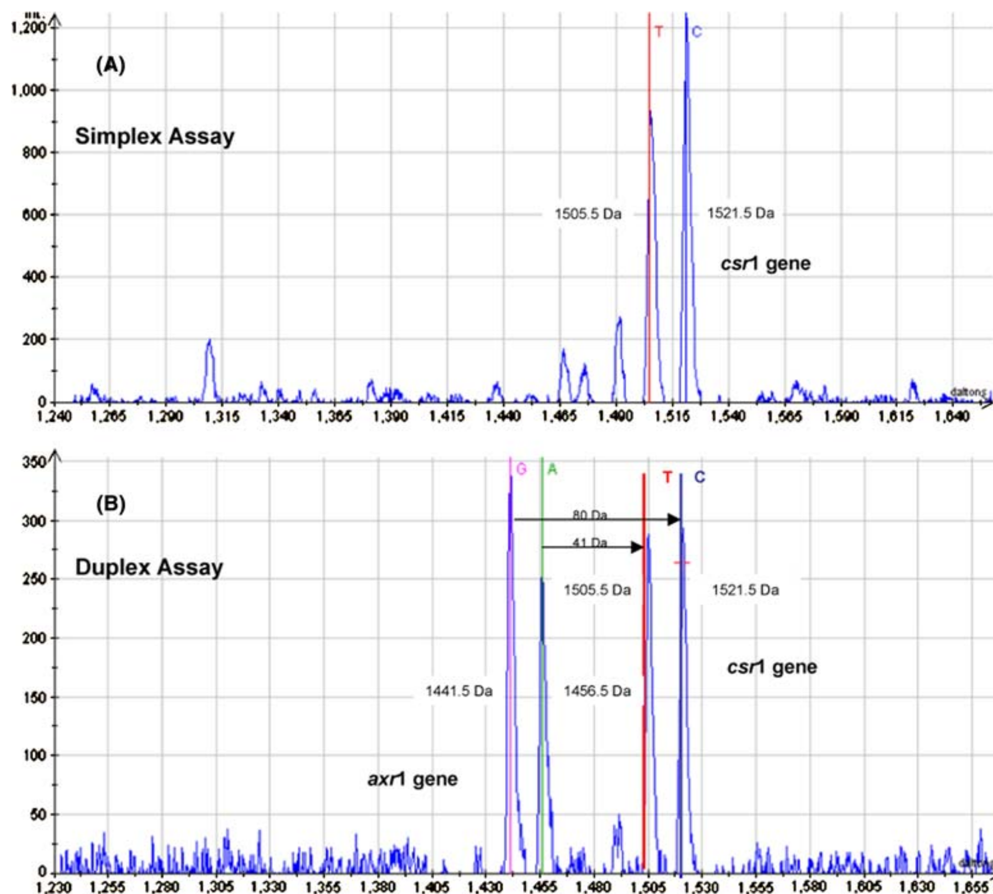

Fig. 1 SNP genotyping by the GOOD assay. The *y*-axis indicates absolute intensity (a.i.) and the *x*-axis indicates the mass in Daltons (*Da*). **a** Simplex analysis for the *CSR* gene from *Arabidopsis thaliana*. The SNP is a T/C polymorphism. This example is a heterozygous individual. The alleles are observed at 1,505.5 Da (*C*) and 1,521.5 Da (*T*). **b** Twofold multiplex genotyping assay for the *CSR* and *AXR1* genes. This duplex example is a double heterozygote. The SNP is a G/A polymorphism in the *AXR1* gene. The alleles are observed at 1,456.5 Da (*A*) and 1,441.5 Da (*G*). The *arrows* indicate the minimal and maximal mass window between primer extension products

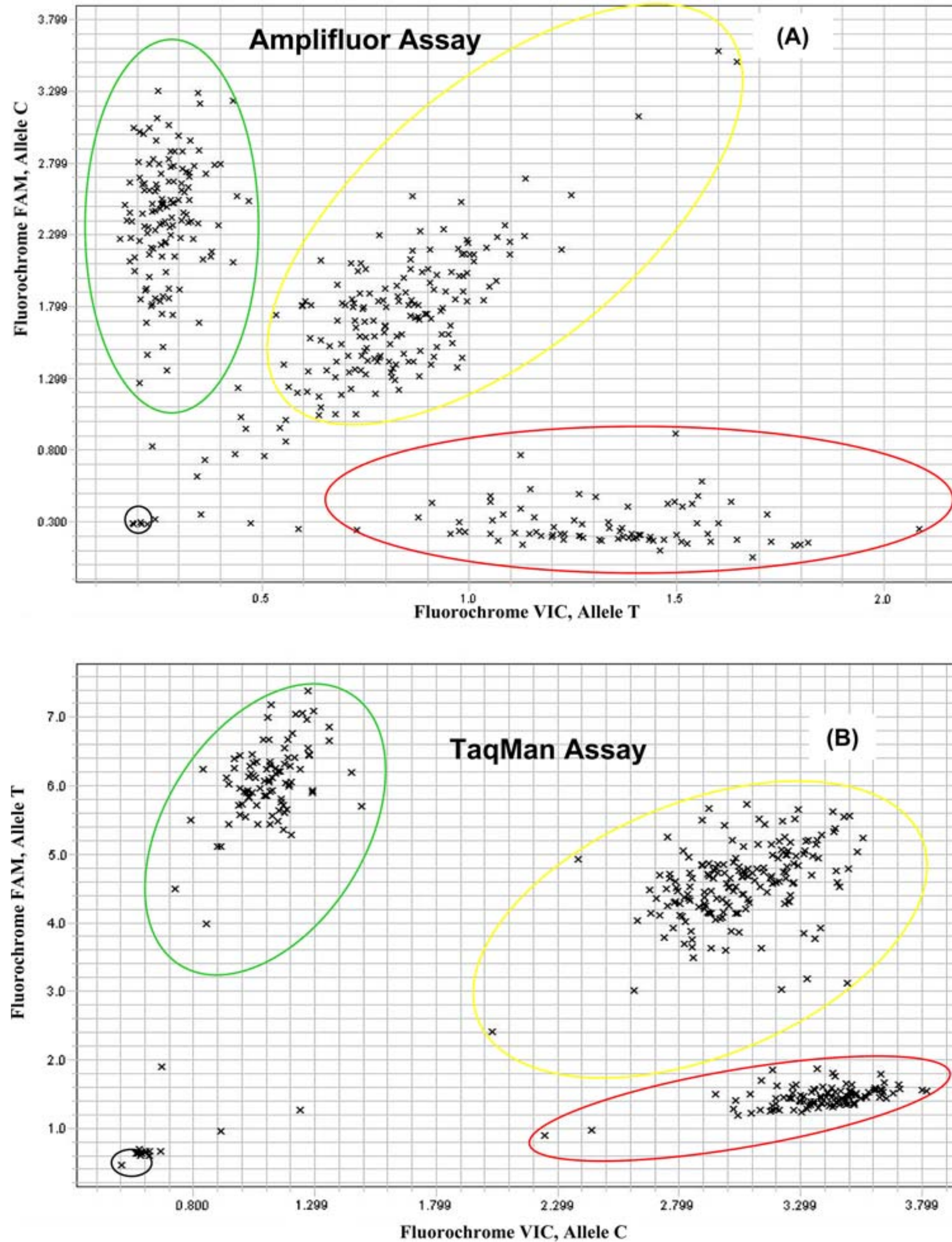

Fig. 2 SNP genotyping by fluorescence methods. The SNP in the *CSR* gene is a T/C polymorphism. **a** Amplifluor® assay. The *x*-axis is the fluorescence data from the VIC-allele (*T*), whereas the *y*-axis is the fluorescence data from the FAM-allele (*C*). The *circle* along the *y*-axis includes C genotypes, the circle along the *x*-axis includes T genotypes and the *middle circle* includes heterozygous samples. The negative controls (water) are inside the circle near the origin. The *points without circles* indicate indeterminate samples. **b** TaqMan® assay. The *x*-axis is the fluorescence data from the VIC-allele (*C*), whereas the *y*-axis is the fluorescence data from the FAM-allele (*T*). The *circle* along the *x*-axis includes C genotypes, the *circle* along the *y*-axis includes T genotypes and the *middle circle* includes heterozygous samples. The negative controls (water) and the indeterminate samples are represented as in the Amplifluor® scattergram

fluorescence) in the Amplifluor® assay was indeed correlated with the lowest DNA concentration and higher fluorescence with higher DNA concentration (correlation factor: $r^2 = 0.99$; $P = 0.002$).

When we used Amplifluor with the *IXR1* gene, a single cluster was obtained in the position where heterozygotes are normally found. Apparently, in this case, there was no specific hybridization and no allelic discrimination occurred. Furthermore, no other primer could be designed for this SNP using the ''Assay-Architect''.

The TaqMan® results for the *CSR* gene are shown in Fig. 2b and Table 1. The good separation obtained between the homozygous and heterozygous clusters in the fluorescence image indicates that there was no non-specific hybridization. Most data points were distant

from the negative controls. These results were thus less sensitive than Amplifluor® to the variation in DNA quantity among individuals, which could indicate a higher yield in the PCR reaction using the TaqMan® technology. A low level of discordance was obtained with the *CSR* gene using TaqMan®; the reason for this is unclear and no homologous sequences were detected in the *Arabidopsis* genome.

Using Amplifluor® and TaqMan®, the overall success rate was over 90% in nearly all cases. To achieve a similar level of success with mass spectrometry, both the normal and the reorganized plates are required. This is because each single plate only gave between 70 and 80% successful data. In general, a low percentage of discordance (<0.5%) was found for each of the three methods. The only exception was the *CSR* gene analyzed by mass spectrometry. As discussed above, we eliminated the possibility that this was due to problems of the DNA samples. For mass spectrometry, the discordant results might be partly explained by contamination due to the numerous successive robot movements. Although the volume of the reaction is small, similar results were obtained when the reactions were scaled up, thus suggesting that the reaction volume is not a critical factor.

Duplex analysis by mass spectrometry

Duplex PCR and primer extension reactions were performed for mass spectrometry. In the duplex PCR protocol, two loci from *A. thaliana*, *AXR1* and *CSR*, were amplified simultaneously. Gene-specific PCR primers were developed to produce different length fragments (*AXR1*: 500 bp and *CSR*: 860 bp, see Supplementary Material Table 1) under the same PCR conditions thus allowing the visualization of the PCR products in an agarose gel. The primer extension products were also designed to be of sufficiently different masses to distinguish each product from the others and the undigested primer. Four α-S-ddNTPs were included in the primer extension reaction to detect all alleles of the two SNPs studied. Figure 1b shows a heterozygote for *AXR1* (allele G: 1,441.5 Da and allele A: 1,456.5 Da) and for *CSR* (allele T: 1,505.5 Da and allele C: 1,521.5 Da).

Application to other plant SNPs

In addition to the comparison described earlier, in the course of a number of research projects, we have genotyped a large number of other SNPs in *Arabidopsis* as well as in other diploid or polyploid plants. Initially, the only technology available was the GOOD assay, thus explaining the large number of results obtained with this technology. Subsequently, as Amplifluor and TaqMan became available, we were able to repeat some experiments which were not successful with the GOOD assay.

Further validation of our results was obtained by comparing the three technologies for a set of markers in *A. thaliana* (used for estimating recombination frequency, see Table 2). For the GOOD assay, 24 of 36 (67%) markers were successfully developed with an average of 92% successful data points (maximum 98.4%, minimum 81.7%). For Amplifluor, 9 of 15 (60%) markers were successfully developed with an average of 96.5% successful data points (maximum 97.7%, minimum 95.2%). For TaqMan, 40 of 42 (95%) markers were successfully developed with an average of 97.3% successful data points (maximum 99.7%, minimum 89.8%). Currently, TaqMan is used as a first choice. These results are summarized in Table 2.

Polyploid species analysis by mass spectrometry

Plants present particular genotyping challenges because a large percentage of plants are polyploid. The first step before sequencing or genotyping thus is to define "gene and genome-specific" primers.

Rapeseed *(Brassica napus)* is considered to be the result of the addition of two parental diploid genomes: *B. oleracea* and *B. rapa*. Accordingly, two or more homeologous genes are found in rapeseed (Brunel et al. 1999). Here, we present the example of the *ADH_2* gene which was genotyped by mass spectrometry. In this case, the SNP is an A/C polymorphism (allele A: 1,465.5 Da and allele C: 1,441.5 Da). The correct analysis of genotypes was confirmed using eight previously sequenced DNA controls. Ninety-nine percent successful results were obtained when the normal and reorganized plates were analyzed and the remaining 1% were indeterminate results. Indeterminate results were the same in both plates, suggesting possible DNA degradation. In our laboratory, additional SNPs from rapeseed and wheat genes were successfully analyzed using the GOOD assay (see Table 2).

Discussion

At the Centre National de Génotypage (Evry, France) a number of high-throughput genotyping technologies are used, primarily for the exploration of the genetic bases of human disease. These include the GOOD assay, pyrosequencing, Amplifluor®, TaqMan®, SNPlex®, Illumina® and Affymetrix®. For the purpose of genotyping plant samples, we required a reliable, relatively rapid method for medium-throughput genotyping.

We have compared three different genotyping technologies for use in medium- to high-throughput applications in plants. Our cumulative results demonstrate that all three technologies, mass spectrometry using the GOOD assay, Amplifluor® and TaqMan®, were successful in discriminating alleles. Although TaqMan® gave the best results under our laboratory conditions, several factors must be considered in assessing which method to use for a given application (Table 3).

Table 2 Summary of plant genotyping results to date

Species	Ploidy	Total SNPs	Method			Genes (fragments)[a]	Individuals genotyped	Application	References
			GOOD assay (% successful marker development)	Amplifier (% successful marker development)	TaqMan (% successful marker development)				
A. thaliana	Diploid	7	5 (71%)	0	1 (50%)	1(2)	1,536	Flowering precocity	V. LeCorre et al., unpublished data
A. thaliana	Diploid	13	10 (77%)	NA	NA	7	384	Genes used to define core collection	McKhann et al. (2004)
A. thaliana	Diploid	36	24 (67%)	NA	NA		768	Recombination frequency	Drouaud et al. (2006)
		15	NA	9 (60%)	NA				
		42	NA	NA	40 (95%)				
A. thaliana	Diploid	8	NA	NA	8 (100%)	2(8)	384	Drought stress	H.I. McKhann et al., unpublished data
Pisum sativum	Diploid	2	1 (50%)	NA	0	1	384	Plant architecture	C. Rameau et al., unpublished data
Alopecurus myosuroides	Diploid	5	5 (100%)	NA	NA		12,639	Herbicide resistance	C. Délye et al., unpublished data
Zea mays	Diploid	62	46 (74%)	NA	NA	11(17)	384	Flowering precocity, grain quality	D. Manicacci, A. Charcosset et al., unpublished data
Brassica napus	Amphidiploid	48	30	NA	NA	30	1,152	Mapping	D. Brunel et al., unpublished data
Triticum aestivum	Hexaploid	25	18 (72%)	NA	NA	7	384	Bread quality	Ravel et al. (2004)

NA not applicable, method was not utilized

[a]Number of fragments is indicated in parenthesis if different from gene number

Table 3 Comparison of SNP genotyping methods

Protocol	Principle	Allele discrimination	Multiplex capacity	Need for replicates	Flexibility in primer design	Number of steps
GOOD assay	Primer extension	Unambiguous	High	Yes	Great flexibility	5
Amplifluor	Allele-specific PCR	Good	None	No	No flexibility	1
TaqMan	Allele-specific hybridization	Good	None	No	Some flexibility (Primer Express)	1

The GOOD assay

Considering the accuracy of the three methods, mass spectrometry offers a clear advantage in that the separation of alleles based on the molecular weight of the primer extension product is unambiguous. The GOOD assay also offers flexibility in primer design as well as the capacity for multiplexing. For the GOOD assay, the experimenter designs his own primers. It is possible to design primers when there are adjacent SNPs with the caveat that the four nucleotides upstream of the SNP must be devoid of SNPs. Another advantage of this technology is that a wide range of PCR fragment sizes can be used, from about 100 bp up to 1.2 kb (unpublished data). This is useful for polyploid species where one wishes to separate homeologous genes. When designing primers, the masses of the primer extension products can be selected so that different products are distinguished in multiplex reactions, and eventual problems can be diagnosed, for example, poor phosphodiesterase digestion or alkylation. Furthermore, it is possible to modify the number of phosphorothioates or the charge tag in order to better distinguish between alleles in the case of multiplex reactions. The GOOD assay also provides the possibility for multiplexing. We have shown here an example of a successful duplex reaction and our results indicate that multiplex genotyping of more loci, previously described in humans (Ross et al. 1998) is extendable to plants.

Another advantage of the GOOD assay is the software available to analyze the results. For the mass spectrometer, the manufacturer provides a program called Genotools Manager which allows the assignment of genotypes and performs a quality control. It does not allow the correction of genotypes or the comparison of several runs. To overcome this limitation, a program was developed for internal use at the Centre National de Genotypage, called SNPMaster, which allows for the analysis of quality control and the genotype. This program is more flexible in that several plates can be compared automatically and consensus results can be exported.

The GOOD assay does suffer a number of serious drawbacks however. It necessitates the analysis of replicates and there is a higher percentage of discordant and missing data points (especially for the *CSR* gene). The GOOD assay involves a large number of steps: PCR, shrimp alkaline phosphatase, primer extension, phosphodiesterase and finally alkylation. Lower success rates may be because this method requires successive reagent additions that make it more vulnerable to pipetting errors and contamination.

Another critical point is the deposition of the matrix on the plate. The extremely high percentage of indeterminate results sometimes obtained with mass spectrometry (18% in the case of the *AXR1* gene) could indicate a problem with the matrix. It is very difficult to control the uniformity of the matrix from one lot to the next. It is likely that small variations in the matrix may give rise to indeterminate results.

Amplifluor® and TaqMan®

Our results here indicate that for both TaqMan® and Amplifluor®, the sample success rate in general is very high (Table 1). Further, the repeatability is high, thus analysis of a single plate suffices. The percentage of indeterminate results was low with both TaqMan® and Amplifluor®.

After an assay has been established, an important factor for medium- or high-throughput production is the time and the number of steps required to successfully genotype a given number of samples. For both Amplifluor® and TaqMan®, a single PCR step is necessary, largely eliminating the possibility of errors. Problems of contamination are small since wells are sealed after the addition of reagents and are never opened again. These methods are more robust, thus there is no longer a need to use a ''normal'' and ''reorganized'' plate. Thus, the fluorescent technologies are able to give faster results. On the other hand, multiplexing is not possible with these techniques. We have also assessed the quality and quantity of DNA that is required to give successful results with the three methods. We did remark that Amplifluor® seems to be more sensitive to low quantities of DNA. Discordant and indeterminate results using the GOOD assay may also be due in part to low DNA quantity.

In both fluorescent techniques, allelic discrimination depends on hybridization in which a single base varies; and in certain cases, nonspecific hybridization means that some data points cannot be assigned with certainty. An extreme example of this was seen using Amplifluor® with the *IXR1* gene for which no individual could be assigned a genotype. While it is true that in certain cases, another primer couple could yield the expected results, constraints in primer design, particularly for

Amplifluor® and TaqMan®, sometimes preclude this possibility (as was the case for the *ixr1-2* SNP).

Concerning primer design, the fluorescent technologies are less flexible than the GOOD assay. Amplifluor® technology requires submitting the sequence with the polymorphism to genotype to a web site, which then provides appropriate primers when possible. There can be no adjacent polymorphisms in the region to which the primer hybridizes (± 20 bases). We have remarked that certain primers chosen using the "AssayArchitect" software may be flawed due to the presence of excessive secondary structure. For TaqMan®, probes and primers are developed by Applied Biosystems by design, but with the constraint that there are no adjacent polymorphisms within two nucleotides of the SNP to be genotyped. A second possibility exists for TaqMan® which is a program called PrimerExpress (Applied Biosystems). Using this software, it is possible to design one's own primers, thus allowing slightly more flexibility and in particular for developing probes and primers that are gene and genome specific, thus theoretically allowing genotyping of polyploid species. It is also possible to design primers that include a second SNP or that give a larger PCR product than is specified using Primer Express (our unpublished results). For the two fluorescent methods, fragment length is restricted to 150 bp or less when using the primer design software AssayArchitect or Applied Biosystems by design. This constraint could limit their applicability to polyploid species. For these two techniques, it is critical to perform a BLAST with the region adjacent to the SNP to ensure there are no homologous sequences (or homeologous, in the case of polyploids) since these methods depend on specific hybridization to discriminate the alleles.

For Amplifluor® and TaqMan®, there is an analysis program that comes with the ABI Prism 7900HT. This program is able to analyze one plate at a time, and the results can be exported to an Excel file. However, the comparison between plates must be done by hand, using copy and paste functions. It is possible to perform the analysis either automatically or manually, thus offering flexibility. For both methods, data analysis is performed by clustering, thus a genotype is only called in the context of the other results. For mass spectrometry, individual samples are called independently of other results.

We have shown that three genotyping technologies, each based on a different principle for SNP detection, can be adapted to plant species, including those that are polyploid, given that "gene and genome specific" primers are available. All three methods have been shown to give satisfactory results for the majority of SNPs tested, thus how can this choice be made? It is virtually impossible to speak of cost since the cost depends on many factors, including the throughput, the labor required, the initial investment in equipment as well as the cost of reagents/kits and the discounts available to a laboratory. Thus, perhaps the key question is the throughput required. At this time in our laboratory, most plant applications entail populations of 1,000 individuals or less with few SNPs. Thus, the throughput could be considered medium. This is in contrast to the genotyping of human populations where 20,000 or more individuals may be genotyped. TaqMan® and Amplifluor® have the advantages of being simple protocols that are transferable between labs and that give a high success rate. In our experience, TaqMan® showed greater specificity and more flexible primer design, while giving a high success rate and thus is the method of choice in our laboratory for this level of throughput. Figure 3 illustrates schematically the appropriate technologies for a given number of individuals and SNPs. As SNPs become increasingly utilized in plant research, higher throughput platforms, like SNPlex and Illumina, will be necessary. We have previously developed SNPlex panels of 48 SNPs for mapping of *Arabidopsis* recombinant inbred lines (unpublished results) and the possibility of

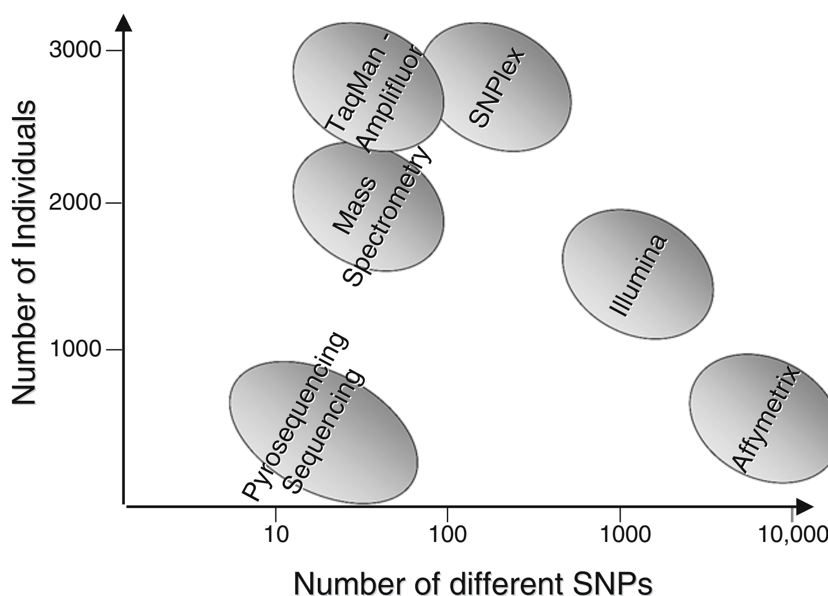

Fig. 3 Examples of several genotyping methods with the corresponding throughput with respect to the number of individuals (*y*-axis) and the number of different SNPs (*x*-axis). TaqMan®, SnaPshot® and SNPlex® are available from Applied Biosystems and Amplifluor® from Serological Corp. Note that with decreasing costs of sequencing, in some cases sequencing of hundreds of individuals remains an option

using Illumina for panels of 384–1,536 SNPs is becoming increasingly desirable to study genome-wide genetic variation in a given species.

Acknowledgements We thank the Mark Lathrop and the members of the Technology Development group of Ivo G. Gut at the Centre National de Génotypage: D. Lechner, C. Besse, F. Boussicault, D. Derbala, J-G. Garnier, F. Mauger, J-A. Perrier, C. Plançon and V. Sourice. We also thank the Informatics group: N. Wiart and F. Dos-Santos. We thank Christine Mézard for providing data on success rates. Part of this study was supported by a grant to FR from Bayer Crop Sciences. Genotyping has been financed by AIP-INRA "Séquençage végétal et post-séquençage" as well as by INRA SPE projects.

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