Quantitative genotyping of single-nucleotide polymorphisms by allele-specific oligonucleotide hybridization on DNA microarrays

Andreas M. Rickert*, Agim Ballvora*[1], Ulrich Matzner†, Manfred Klemm† and Christiane Gebhardt*

*Max-Planck-Institute for Breeding Research, Carl-von-Linne-Weg 10, 50829 Cologne, Germany, and
†Alpha Technology GmbH, Hürth, Germany

Genotyping of SNPs (single-nucleotide polymorphisms) has challenged the development of several novel techniques [Twyman and Primrose (2003) Pharmacogenomics **4**, 67–79]. Most of these methods have been introduced to discriminate binary SNPs in diploid species. In the present study, the quantitative genotyping of SNPs in natural DNA pools of a polyploid organism via DNA microarrays was analysed. Three randomly selected SNP loci were genotyped in the tetraploid species potato (*Solanum tuberosum*). For each SNP, 24 oligomers were designed, 12 with forward and 12 with reverse orientation. They contained the polymorphic site at one of the positions 11, 14 and 17. Several steps of optimizations were performed, including the 'materials' used and the establishment of hybridization conditions. Glass surfaces were either epoxy- or aldehyde-modified, and allele-specific oligonucleotides contained either SH or NH_2 groups. Hybridization stringency conditions were established by varying the concentration of formamide in the hybridization buffer. For SNP BA213c14t7/403, the quantitative discrimination between all four different naturally occurring genotypes could be demonstrated.

Introduction

SNPs (single-nucleotide polymorphisms) represent the most common type of genetic variations accounting for approx. 90% of all polymorphisms [2]. As a result of genome sequencing efforts, large number of SNPs have been discovered, not only in humans [3] (see also the SNP Consortium LTP at http//snp.cshl.org), but also in other diploid organisms such as *Drosophila melanogaster* (fruitfly or vinegar fly) [5], *Arabidopsis thaliana* (thale cress) [6] and cereal species [7], and recently in the polyploid potato *Solanum tuberosum* [8]. Owing to their direct accessibility, SNPs have become popular markers for a wide range of genetic applications, and a number of different SNP genotyping methods have been introduced. However, no single method has been widely accepted [1,9–14]. For linkage and association studies, many SNP markers have to be scored in large number of individuals. Since this procedure is time-consuming and costly, an alternative approach is to pool samples of affected and unaffected individuals and subsequently estimate, quantitatively, allele frequencies in these subsets. To attain this goal, several SNP genotyping methods have been evaluated [15–28]. Although ASO (allele-specific oligonucleotide) hybridization on microarrays is among the most promising techniques for genotyping individual SNPs [6,29], documented studies of its applicability for quantification of allele frequencies in DNA pools are lacking. In the present paper we describe quantitative genotyping using microarray-based ASO hybridization of three binary SNPs in different homozygous and heterozygous tetraploid potatoes. These genotypes represent natural pools of four alleles for which five allelic ratios are distinguishable: 4:0, 3:1, 2:2, 1:3 and 0:4 [25].

Materials and methods

Plant material

F1 plants of a cross between two tetraploid potato cultivars SR11 and SR12 were provided by SAKA-RAGIS (Pflanzenzucht GbR, Windeby, Germany) [8]. F1 plants that had been evaluated by pyrosequencing or SNuPE (single nucleotide primer extension) combined with DHPLC (denaturing high-performance liquid chromatography) for the test of SNPs and represented the different allele dosages, were selected for the evaluation of quantitative accuracy of allele-specific hybridization on microarrays. Genomic DNA was isolated from leaf tissue as described previously [30].

SNP markers

The SNPs BA44a10t7/174, BA213c14t3/268 + 269 and BA213c14t7/403 have been identified by comparative Sanger

```
      3'-GTCAACGTAGGNAGTCGACGAA-tail
         3'-AACGTAGGNAGTCGACGAAACA-tail          } ASOs querying
            3'-GTAGGNAGTCGACGAAACAGAA-tail          sense strand

5'-...CTGCTTTTGCAGTTGCATCCWTCAGCTGCTTTGTCTTTCT...-3'  } Target sequence
3'-...GACGAAAACGTCAACGTAGGWAGTCGACGAAACAGAAAGA...-5'

           tail-AGTTGCATCCNTCAGCTGCTTT-3'
         tail-TGCAGTTGCATCCNTCAGCTGC-3'            } ASOs querying
       tail-TTTTGCAGTTGCATCCNTCAGC-3'                antisense strand
```
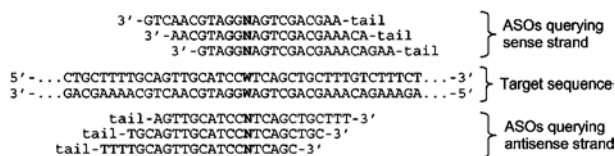
Figure 1   Schematic representation of the panel of ASOs designed for genotyping the A ↔ T-SNP BA213c14t7/403

The set of ASOs for allele-specific hybridization comprises 24 different 22-meric oligonucleotides querying the sense as well as the antisense strand of the PCR-amplified target region including the SNP. ASO probes are walking over the polymorphic sites presenting the allelic nucleotide at position 11, 14 or 17 from the 5′-end. For each of these ASOs, four different versions were designed, carrying all four bases at the SNP position. ASOs are attached to tails composed of a C_6 linker and an SH or NH_2 group. N, A/T/G/C; W, A/T.

DNA sequencing and can be accessed through the website http://gabi.rzpd.de/PoMaMo.html [8]. The precise allele dosages of SNPs in the different genotypes were confirmed by pyrosequencing [25] or by SNuPE in combination with DHPLC as described previously [31].

Amplification and labelling of target sequences

For amplification and labelling of target sequences (lengths between 180 and 216 bp), the following primer combinations were used: BA44a10t7Fa1 (5′-CTACATCACCTGC-AGCACTCC-3′) and BA44a10t7Ra1 (5′-TTTCATGGAA-GCTGGACGGCTAG-3′); BA213c14t3Ca1 (5′-CGGTCA-GGGACATCATGCGCA-3′) and BA213c14t3Da1 (5′-GT-GGTATCTGCTAGCAATTGCTAC-3′); BA213c14t7Ba1rev (5′-TCTAACCTGGGCAGTCAGAGAC-3′) and BA213-c14t7B (5′-TCTTGACGCAAACCTCTGCGAG-3′). Primers were purchased from Metabion (Martinsried, Germany). PCRs were performed as described in [25]. Aliquots (30 ng) of the initial PCR amplifications were subjected to a second round of PCR containing 20 μM Cy3 (indocarbocyamine)-dUTP (Amersham Biosciences, Freiburg, Germany) and 80 μM dTTP. PCR products were purified using a PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Preparation of arrays

For each SNP, 24 different 22-meric oligonucleotides were designed: 12 for the forward-strand sequence and 12 for the reverse-strand sequence. All four bases were represented at the polymorphic site, which was at position 11, 14 or 17 from the 5′-end (Figure 1). Each oligonucleotide probe (10–20 μM solution) was spotted in triplicate/slide using the spotting device BioGrid (BioRobotics, Oxford, U.K.). The probes were covalently attached to the glass slides (Quantifoil, Jena, Germany) following the manufacturer's instructions. The surfaces of the slides were either epoxy-modified or aldehyde-modified. The probe-loaded slides

were blocked directly before hybridization following the manufacturer's (Quantifoil) instructions.

The ASOs had C_6 linkers at their 5′-ends and were either SH- or NH_2-modified, and were purchased from Inter-activa Biotechnologie GmbH (Ulm, Germany).

Hybridization and washing of arrays

Labelled PCR product (20 μl) from the targeted locus was mixed with 180 μl of hybridization buffer [10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 0.6 M NaCl, 1 × Denhardt's solution (0.02 % Ficoll 400, 0.02 % polyvinylpyrrolidone and 0.02 % BSA), 0.1 mg/ml herring sperm DNA and 0–50 % form-amide]. Different formamide concentrations (0, 10, 20, 30, 40 or 50 %) were tested to determine the most appropriate stringency conditions. The hybridization cocktail was de-natured at 95 °C for 5 min, cooled on ice, applied to the array using a hybridization frame (GeneScan, Freiburg, Germany) and incubated at 42 °C for 4 h. Subsequently, the slide was washed for 10 min with 10 mM Tris (pH 7.5) and 39 mM NaCl at 32 °C, and then for 10 min with 10 mM Tris (pH 7.5) and 19.5 mM NaCl at 32 °C.

Microarray reading and data analysis

Arrays were scanned on the GenePix™ 4000B scanner (Axon Instruments, Foster City, CA, U.S.A.) and images were analysed using the software GenePix ™ Pro (Axon Instruments).

Results and discussion

The ASO hybridization on glass slides required several opti-mization steps [14]. Epoxy- or aldehyde-modified glass sur-faces were tested with the same ASOs and hybridization conditions. The signal intensities and signal-to-noise ratios were comparable for both types of slides (results not shown). However, the use of alternative 5′-modifications of ASOs affected the hybridization results substantially. 5′-SH-modified oligomers produced up to 35.4-fold stronger signals when compared with the same oligonucleotides having an NH_2-modification. No difference in the signal-to-noise ratio was detectable (results not shown).

Owing to the difficulty in predicting hybridization con-ditions and sequences of ASOs that will allow optimal allele discrimination, these parameters had to be found empirically [10]. To select the appropriate oligonucleotide sequence, the strategy of probe redundancy [29] was chosen. Panels of ASO probes containing the polymorphic site at various positions and querying the forward as well as the reverse-strand sequences were used for each SNP (Figure 1). In con-trast with ASO probes containing the polymorphic site at nucleotide position 17 from the 5′-end, ASOs with the allele-specific nucleotide at position 11 or 14 from the 5′-end
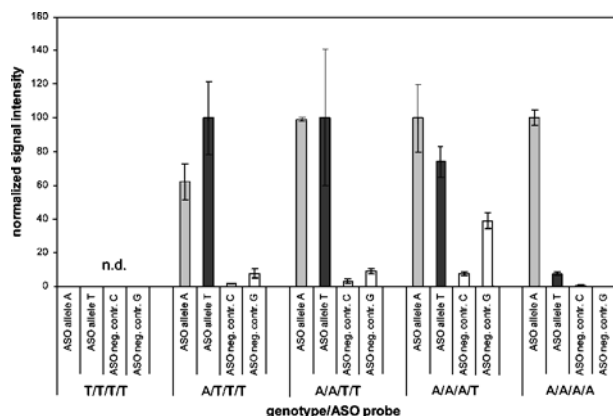
Figure 2   Quantitative genotyping results of four different genotypes of the A ↔ T-SNP BA213c14t7/403

Results are shown for the optimized hybridization condition and ASO probes that allowed the best discrimination between the alleles (hybridization buffer containing 50% formamide; antisense ASO with polymorphic site at nucleotide position 15 from the 5′-end). The heights of the histograms correspond to the normalized fluorescence intensities of the spots. S.D. values were calculated on the basis of signal intensities of the three replicas spotted for each ASO probe. The allelic nucleotides are highlighted in black and grey respectively. The genotype T/T/T/T was not available.

were both appropriate to discriminate between different alleles. Sense and antisense oligonucleotides were tested to determine the probe orientation that allowed better allele discrimination. For the SNP locus BA213c14t7/403, signals of the same quality were obtained with both sense and antisense strands, whereas, in the case of BA213c14t3/268 + 269, allele discrimination was only possible with the antisense ASOs (results not shown).

The hybridization conditions appeared to be the most important parameter requiring optimization. Several formamide concentrations in the hybridization buffer were tested to achieve a strong and specific hybridization signal with a low background. It turned out that each SNP locus required specific stringency conditions for optimal results. The best hybridization results were obtained using 10% (v/v) formamide for BA44a10t7/174, 30% formamide for BA213c14t3/268 + 269 and 50% formamide for BA213c14t7/403. The requirement to establish specific hybridization conditions for each SNP has been considered as a major drawback of ASO hybridization on microarrays [14].

For SNP loci BA213c14t3/268 + 269 and BA213c14t7/403 the signal-to-noise ratio was sufficiently low for genotyping the targeted polymorphisms. For SNP BA213c14t7/403, all four different genotypes, which were available, could be quantitatively discriminated (Figure 2). In case of BA213c14t3/268 + 269, the expected hybridization signals for the two homozygous genotypes were obtained, but the quantitative accuracy failed to distinguish the different allelic ratios of the three heterozygous genotypes (results not shown).

For SNP BA44a10t7/174, the conditions used were not stringent enough to achieve a reliable genotyping (results not shown). The signals obtained from the negative controls (ASO probes representing non-existing allelic bases) did not allow robust genotyping. For this marker it seems necessary to improve other hybridization conditions such as temperature and, possibly, primer design.

In conclusion, our results suggest that it is possible to perform quantitative genotyping of SNPs using allele-specific hybridization on microarrays. Individual setting of specific hybridization conditions for each SNP and selection of appropriate material are two prerequisites to obtain reliable genotyping results. To our knowledge, this is the first attempt to show the feasibility of the method for quantitative genotyping of SNPs. A comparison of advantages of this method with other techniques will only be possible when more examples will be available. Most of the methods tested so far for quantitative genotyping of SNPs [25] inherit their drawbacks. Therefore the procedure described here remains a promising alternative particularly for simultaneous analysis of a large number of SNPs.

References

1   Twyman, R. M. and Primrose, S. B. (2003) Pharmacogenomics **4**, 67–79

2   Collins, F. S., Guyer, M. S. and Chakravarti, A. (1997) Science **278**, 1580–1581

3   The International SNP Map Working Group (2001) Nature (London) **409**, 928–933

4   Reference deleted

5   Long, A. D., Lyman, R. F., Langley, C. H. and Mackay, T. F. C. (1998) Genetics **149**, 999–1017

6   Fan, J.-B., Chen, X., Halushka, M. K., Berno, A., Huang, X., Ryder, T., Lipshutz, R. J., Lockhart, D. J. and Chakravarti, A. (2000) Genet. Res. **10**, 853–860

7   Buckler, E. S., Thornsberry, J. M. and Kresovich, S. (2001) Genet. Res. **77**, 213–218

8   Rickert, A. M., Jeong, H. K., Meyer, S., Nagel, A., Ballvora, A., Oefner, P. J. and Gebhardt, C. (2004) Plant Biotechnol. J. **1**, 399–410

9   Ellis, M. C. (2000) Genet. Res. **10**, 895–897

10   Gut, I. G. (2001) Hum. Mutat. **17**, 475–492

11   Kwok, P. Y. (2000) Pharmacogenomics **1**, 231–235

12 Kwok, P. Y. (2001) Annu. Rev. Genomics Hum. Genet. **2**, 235–258

13 Shi, M. M. (2001) Clin. Chem. **47**, 164–172

14 Syvänen, A. C. (2001) Nat. Rev. Genet. **2**, 940–942

15 Breen, G., Harold, D., Ralston, S., Shaw, D. and St Clair, D. (2000) Biotechniques **28**, 464–470

16 Cho, R. J., Mindrinos, M., Richards, D. R., Sapolsky, R. J., Anderson, M., Drenkard, E., Dewdnay, J., Reuber, L., Stammers, M., Federspiel, N. et al. (1999) Nat. Genet. **23**, 203–207

17 Germer, S., Holland, M. J. and Higuchi, R. (2000) Genet. Res. **10**, 258–266

18 Giordano, M., Mellai, M., Hoogendoorn, B. and Momigliano-Richiardi, P. (2001) J. Biochem. Biophys. Methods **11**, 101–110

19 Gruber, J. D., Colligan, P. B. and Walford, J. K. (2002) Hum. Genet. **110**, 395–401

20 Le Hellard, S., Ballereau, S. J., Visscher, P. M., Torrance, H. S., Pinson, J., Morris, S. W., Thomson, M. L., Semple, C. A. M., Muir, W. J., Blackwood, D. H. R. et al. (2002) Nucleic Acid Res. **30**, e74

21 Lindroos, K., Sigurdsson, S., Johansson, K., Rönnblom, L. and Syvänen, A.-S. (2002) Nucleic Acids Res. **30**, e70

22 Matyas, G., Giunta, C., Steinmann, B., Hossle, J. P. and Hellwig, R. (2002) Hum. Mutat. **19**, 58–68

23 Norton, N., Williams, N. M., Williams, H. J., Spurlöock, G., Kirov, G., Morrris, D. W., Hoogendoorn, B., Owen, M. J. and O'Donovan, M. C. O. (2002) Hum. Genet. **110**, 471–478

24 Olsson, C., Liljedahl, U. and Syvänen, A. C. (2003) Methods Mol. Biol. **212**, 167–176

25 Rickert, A. M., Premstaller, A., Gebhardt, C. and Oefner, P. J. (2002) Biotechniques **32**, 592–593

26 Ross, P., Hall, L. and Haff, S. A. (2000) Biotechniques **29**, 620–629

27 Shifman, S., Pisante-Shalom, A., Yakir, B. and Darvasi, A. (2002) Mol. Cell. Probes **16**, 429–434

28 Zhang, S., Van Pelt, C. K., Huang, X. and Schulz, G. A. (2002) J. Mass Spectrum **37**, 1039–1050

29 Wang, D. G., Fan, J. B., Siao, C. J., Berno, A., Young, P., Sapolsky, R., Ghandour, G., Perkins, N., Winchester, E., Spencer, J. et al. (1998) Science **280**, 1077–1082

30 Oberhagemann, P., Chatot-Balandras, C., Bonnel, E., Schäfer-Pregl, R., Wegener, D., Palomino, C., Salamini, F. and Gebhardt, C. (1999) Mol. Breeding **5**, 399–415

31 Premstaller, A., Oberacher, H., Rickert, A., Huber, C. G. and Oefner, P. J. (2002) Genomics **79**, 793–798