SNP Typing in Cereals: Comparison of SSCP and SNaPShot Markers Using the Barley *Mlo* Locus as a Model

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Abstract: We compared the applicability of SSCP and SNaPShot markers in barley *Mlo* loci as a model of SNP detection in cereals. Whereas the development of SSCP markers required optimisation steps, the ddNTP primer extension (SNaPShot) procedure based on knowledge of target sequence provided expected results without any previous optimisations. We have shown, that SNPs can be easily scored using the ABI PRISM 310 Genetic Analyser.

Keywords: SSCP; SNaPShot; *Mlo*; SNP; DNA marker; barley; primer extension

Barley breeding is mainly focused on yield stability and end-use quality of grains. Information about allelomorphism of genes conditioning such traits is required. Several techniques are suitable for the detection of single nucleotide substitution (SNPs = single nucleotide polymorphisms) (Liljedahl & Syvanen 2002) and short insertions/deletions. SNPs are placed along the chromosomes where the genetic code tends to vary among individuals (Nollou & Wagener 1997).

DNA sequencing is the only method to characterise newly revealed SNPs (Nollou & Wagener 1997). However, DNA sequencing is not a cost and time effective technique for large-scale SNP screening. We describe here the application of two fluorescence-based techniques (Dideoxy Nucleotide – ddNTP) Primer Extension and Single Strand Conformation Polymorphism – SSCP) for SNP detection using the genetic analyser ABI PRISM 310 (Perkin Elmer).

SSCP analysis is an accepted and useful application for the detection of mutations based on the ability of a single or multiple nucleotide change in the sequence to alter the electrophoretic mobility of a DNA single strand under non-denaturing conditions (Orita *et al*. 1989).

The ddNTP primer extension is based on the unlabeled primer extension by a dideoxy nucleotide. The oligonucleotide primer is designed to hybridise to the DNA-template adjacent to the mutation site. The primer is extended by one base when submitted to the thermal cycling in the presence of DNA polymerase and fluorescence-labelled ddNTPs terminators. The fluorescence-labelled product is readily separated by capillary electrophoresis. Each of the used ddNTPs are assigned to a different fluorescent dye so that different fluorescence signals correspond to different alleles. Two fluorescence signals are detected when both alleles are present in heterozygous state. Applied biosystem developed a streamline protocol for this assay known as SNaPShot.

We have selected well-defined SNPs in the barley *Mlo* gene (Table 1) to illustrate the applicability of these techniques for mutation screening in cereals (breeding lines, genetic resources). The *Mlo* gene was sequenced by Büscheges *et al*. (1997). Mutations of the *Mlo* gene confer resistance to *Blumeria graminis*, an important barley pathogen (Jörgensen 1992).

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MATERIAL AND METHODS

Plant material and DNA isolation. Barley cultivars and lines with defined resistance to powdery mildew were used (Table 1). Seeds were provided by the RICP Gene Bank in Prague-Ruzyně. Genomic DNAs were extracted from young leaves using the protocol of Saghai-Maroof *et al*. (1984).

Sequencing. Exon-1 was amplified by the newly designed forward primer 5′-TTT TTC CTT TCg CCT CTC TTg-3′ and reverse primer 5′-TTT gCg AAC Tgg TAT TCC AAg-3′ under thermocycling conditions as followed: 96°C 1 min, 94°C 1 min, 68–60°C 30 s touch down 18 cycles, 72°C 1.5 min, followed by 20 cycles 94°C 1 min, 60°C 30 s, 72°C 1.5 min with final extension 72°C 5 min. The amplification products were purified using the QIAquick PCR Purification Kit (Qiagen). The sequence reactions of purified PCR products were prepared using the BigDye terminator kit v. 3.0 (Applied Biosystems). The products were sequenced using ABI PRISM 310.

SSCP. The sequence fragment in the *Mlo* exon-1 was amplified by the three different newly designed fluorescence labelled forward and reverse primers. **(1)** F-5′-Cgg gCA Agg AAg gAg gTT gC-3′-FAM and R-5′-AgC TTg Tgg Agg CCg TgT TCC-3′-TET, **product size 154 bp**, **(2)** F-5′-gTg CAT CTg CgT gTg CgT ACC-3′- FAM and R-5′-gCT TgT ggA ggC CgT gTT CC-3′-TET, **product size 200 bp** and **(3)** F-5′-CTC Cgg gCA Agg AAg gAg gTT gC-3′-FAM and R-5′-gCg CAC gCC gAT TAC AAC CAA gg-3′-TET, **product size 242 bp**. The reaction mixture contained 1x Dynex buffer, 2mM $MgCl_2$, 100 ng DNA template and 2U Taq polymerase (Dynex). The PCR thermal condition profile was: 94°C 3 min, 35 cycles of 94°C 1 min, 70°C 1 min, 72°C 1 min with final extension 72°C 5 min. The amplification products were denatured in formamide at 95°C 5 min. Denatured products of each cultivar were separated in the non-denaturing polymer using capillary electrophoresis of the ABI PRISM 310. Several conditions for the SSCP analysis were tested: five different temperatures ranging from 20–45°C, different concentrations of non-denaturing polymer (3% and 5% GSP) and electrophoretic voltage 9 and 13 V respectively.

ddNTP primer extension. The SNaPShot procedure was applied for the *mlo*9 SNP detection, following the prorotocol of the SNaPShot ddNTP Primer Extension Kit (US patent licence 5,075,216, Applied Biosystems). The Exon-1 was amplified using the same primers as for sequencing. The amplification products were purified as recommended. The forward and reverse *Mlo*9 extension primers R-CgA Cgg CgT CTC Cgg CAg CTC CC and F-ggA CAA AAA Agg ggT gCC ggC were designed and used together with fluorescently labelled ddNTP and Taq polymerase. The extension products were analysed using the ABI PRISM 310.

RESULT AND DISCUSSION

Advanced techniques are applied to screen mutations in human and animal populations (Vignal *et al*. 2002; Weiner & Hudson 2002). Plant breeders require tools for efficient large-scale screening of breeding lines as well. We aimed to evaluate the effectiveness of the SSCP and fluorescent ddNTP primer extension procedure for the detection of mutations in cereals.

Table 1. SNPs in exon 1 of *Mlo* gene defined in barley genetic resources

Cultivar	Resistance	SNP
Amulet	susceptible	
Diamant	susceptible	
mlo-mutant of Carlsberg II*	*mlo*8	$A^{1} \rightarrow G$
Alexis	*mlo9*	$C^{28} \rightarrow T$
Sultane	*mlo*9	$C^{28} \rightarrow T$
mlo-mutant of Plena*	*mlo13*	$T^{89} \rightarrow A$
Grosso	*mlo*11	unknown
Apex	*mlo*11	unknown
KM2161	resistant (*mlo*?)	unknown

**GB = obtained from the Gene Bank Prague-Ruzyně

Five different mutations (Table 1) are known in the exon-1 of *Mlo* (*mlo*-5, -8, -9, -13, -17) (Büscheges *et al*. 1997). To exclude doubts about the authenticity of plant material we proved the presence of mutations in the provided barley genetic resources by sequencing. *Mlo* exon-1 of following cultivars: Atribut, KM2161, a *mlo*-mutant of Carlsberg II, Alexis, Grosso and a *mlo*-mutant of Plena. The presence of the expected mutations was confirmed as well as the homozygocity of the accessions. No mutation was found in the *Mlo* exon-1 of KM2161.

Three fragments corresponding to a part of exon-1 containing point mutations (*mlo*-9, *mlo*-8, *mlo*-13) and the standard *Mlo* allele of the susceptible cultivar Amulet were amplified. The electrophoretic mobilities of single strands, measured as retention time of the amplified standard allele, and mutant alleles were compared. This is the most critical aspect of SSCP. A mutation was supposed, if the electrophoretic mobility of an unknown allele exceeded three times the standard deviation of electrophoretic mobility of the standard allele. This approach allows an effective discrimination of mutated alleles, which appear statistically as out-layers. The mentioned calculations were used Atha *et al*. (2001) and Kozlowski and Krzyzosiak (2001). The effect of mutation in a single strand conformation formation and therefore electrophoretic mobility is unpredictable. Several parameters (GSP concentration, run temperature, run voltage, amplicon size) were therefore tested to develop an optimal protocol for the detection of *Mlo* mutations. We have found the following conditions optimal for the differences in mobility shift between the 242bp (amplicon size) PCR fragments of the *Mlo*-standard and the *Mlo*-mutant allele, respectively, were most significant at 40°C (run temperature) in 3% GSP (GSP concentration) at 13V (run voltage) (Table 2). No changes in mobility were detected in the *Mlo* exon-1 of cv. KM2161 under any tested variants. The ABI PRISM 310 Genetic Analyser is not equipped with a cooling system so that temperatures below 20°C, which are often reported as suitable to reveal mutations (Baba *et al*. 2003), could not be tested. However, as we have shown, some mutations can be detected at temperatures higher than 20°C. The three amplicons with different sizes include all defined *Mlo* mutations. We are able to screen the *Mlo*/*mlo* among selected genotypes using the optimised SSCP protocol, but we can not characterise the type of *Mlo* mutation by the use of SSCP. The ddNTP primer extension assay was used to differentiate SNPs in the *Mlo* locus and to screen genotypes.

We genotyped the *mlo*9 SNP (C/T) using SNAPShot analysis (Table 2). The obtained SNAPShot data corresponded to the sequence analysis (data not shown). The *mlo*9 forward primer was extended by ddCTP or ddTTP and the *mlo*9 reverse primer was extended by ddGTP or ddATP. Different fluorescence signals correspond to individual ddNTP as in the sequence analysis. It enables us to detect

Table 2. Data from *mlo*9 SNP screening using SSCP and SNAPShot analysis

Cultivar	SSCP (*mlo* mutations)		SNAPShot (*mlo*9 mutation)	
	retention time (s)	mobility shift[b]	*mlo*9 forward primer	*mlo*9 reverse primer
Diamant	4135.81	**–2.09**	dd**C**TP	dd**G**TP
Alexis (*mlo*9)	4129.25	–8.65	dd**T**TP	dd**A**TP
Sultane (*mlo*9)	4132.33	–5.57	dd**T**TP	dd**A**TP
*mlo*8-mutant of Carlsberg II	4130.56	–7.29		
*mlo*13-mutant of Plena	4131.46	–6.44		
Amulet	4139.36		dd**C**TP	dd**G**TP
Amulet	4139.98			
Amulet	4136.63			
Amulet	4137.00			
Amulet	4136.28			
Mean of Amulet	4137.85 (1.695[a])			

[a]standard deviation; [b]mobility shift is defined as the difference between retention time of the mutant peak and the average normal peak (cv. Amulet)

both dyes in heterozygous genotypes. The retention times of short extension products (24 bp) during capillary electrophoresis were approximately 11 min per sample. The ddNTP primer extension analysis on the ABI PRISM 310 is a time effective technique in comparison to sequence analysis (60 min per sample) and SSCP (30 min per sample). We verified on the collection of defined barley, that the detection of the *mlo9* mutation using SNaPshot is highly reproducible and reliable. We conclude that the SNaPShot protocol can be easily used to develop SNP markers.

In summary, fluorescence-based ddNTP primer extension and optimised SSCP protocols are simple and versatile techniques for large-scale screening of defined point mutations.

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Abstrakt

Ovesná J., Poláková K., Kučera L., Rulcová J. (2003): **Genotypizace SNP u obilovin: porovnání SSCP a SNaPShot markerů na modelu *Mlo* lokusu ječmene**. Czech J. Genet. Plant Breed., **39**: 109–112.

Porovnávali jsme možnost využití SSCP a SNaPShot markerů pro detekci SNP s využitím lokusu *Mlo* ječmene jako modelu. Zatímco vývoj SSCP markeru vyžadoval optimalizaci několika kroků, SNaPShot metoda založená na využití ddNTP a extenzi primerů dává požadované výsledky bez nutnosti optimalizací. Prokázali jsme, že SNP mohou být snadno detekovány s využitím zařízení pro sekvenční a fragmentační analýzu na ABI PRISM 310.

Klíčová slova: SSCP; SNaPShot; *Mlo*; SNP; DNA marker; ječmen; extence primerů

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