

Received 16 June 2004

Accepted 4 October 2004

DEVELOPMENT OF THE SINGLE NUCLEOTIDE POLYMORPHISM MARKER OF THE WHEAT *Lr1* LEAF RUST RESISTANCE GENE

MIROSLAW TYRKA^{1*}, LIDIA BŁASZCZYK², JERZY CHEŁKOWSKI²,
VOLKER LIND³, ILONA KRAMER³, MARLIS WEILEPP³, HALINA
WIŚNIEWSKA² and FRANK ORDON³

¹Laboratory of Population Genetics, Polonia University, Pułaskiego 4/6, 42-200 Częstochowa, Poland, ²Institute of Plant Genetics, Polish Academy of Sciences, Strzeszyńska 34, 60-479 Poznań, Poland, ³Federal Centre for Breeding Research on Cultivated Plants, Institute of Epidemiology and Resistance, Theodor-Roemer-Weg 4, 06449 Aschersleben, Germany

Abstract: The range of publicly available data on plant nucleotide sequences opens a new possibility in the design of SNP assays. The purpose of this study was to identify point mutations in genomic sequences closely linked to the *Lr1* leaf rust resistance gene, and to develop SNP markers based on primer extension (SNuPE) facilitating efficient marker-based selection procedures, e.g. the pyramiding of resistance genes. Studies were performed on the panel of 37 wheat cultivars, the set of 41 Thatcher near-isogenic lines of spring wheat and on the 21 individuals derived from doubled-haploid (DH) lines derived from 'Henika' (*Lr1*) x 'IPG-SW-14'. A minisequencing reaction run with Lr1_98F primer detected four genotypes (T, C+T, C and "null") in the set of all *Triticum aestivum* varieties tested. In this study, it turned out that the T allele is associated with the *Lr1* gene in a wide genetic background.

Key Words: Wheat, SNP, Single Nucleotide Polymorphism, Leaf Rust, Minisequencing

INTRODUCTION

Leaf rust caused by *Puccinia recondita* f. sp. *tritici* is a significant pathogen of common wheat. An infection of this fungal disease can lead to severe yield losses, so growing resistant cultivars is an economic and environmentally friendly approach to controlling it. More than 50 leaf rust resistance genes have been described [1]. The *Lr1* gene, conferring partial resistance, was identified in the variety Malakoff [2] and located on chromosome 5DL [3]. Virulence to *Lr1*

*Corresponding author; tel: +48 (034) 3684233, fax: +48 (034) 3249662,
e-mail: mtyrka@ap.edu.pl

is common in North America and is present at different levels in European countries [4]. Therefore, *Lr1* has to be considered useful, especially in combination with other *Lr* genes, e.g. in strategies of gene pyramiding [4]. At present, the sequence tagged site (STS) marker of *Lr1* is of restricted usefulness in breeding as it is not specific in 50% of the cultivars tested [5]. This implies the need for a robust and specific assay for the detection of *Lr1*. High resolution mapping led to the location of *Lr1* in a 0.16 cM region flanked by two RFLP markers, *Xabc718* and *Xpsr567* [6]. This opens the opportunity to use published sequence data for the identification and detection of single nucleotide polymorphism (SNP) markers.

A range of methods have been developed for SNP detection. The RFLP method can be considered the first used for the detection of mutations in restriction sites. Since the 1980s, a variety of more or less sophisticated methods have been developed to screen for single nucleotide polymorphism. The methods of SNP analysis can rely on enzymatic cleavage, hybridization with allele-specific probes, oligonucleotide ligation or single nucleotide primer extension, and can exploit different resolution and detection methods [7]. Allele-specific amplification, ligase chain reaction (LCR), heteroduplex analysis, dideoxy sequencing, pyrosequencing, single-nucleotide primer extension, real-time PCR analysis and microarray scanning remain the methods of choice, with different throughputs and costs of a single assay [8, 9].

Direct sequencing of PCR products resulted in the discovery of 1 SNP per 31 bp of non-coding maize regions; this frequency was lower (1 per 124 bp) in coding regions [10]. One SNP per 540 bp was detected in wheat coding regions [11]. The abundance of SNPs in cereals makes them extremely important for creating high density genetic maps. The range of public databases of nucleotide sequences opens the possibility to design SNP assays for genes or sequences of interest. Projects of SNP detection and validation are run for maize (<http://www.agron.missouri.edu/>) and wheat (<http://wheat.pw.usda.gov/ITMI/WheatSNP/>). In wheat, SNPs from ESTs are predominantly being identified in the frame of the International Wheat SNP Consortium. Pyrosequencing was frequently used in cereals [10, 12-15]. This method has been used for SNP discovery in regions flanking microsatellite markers, and for validation of SNPs identified in the EST database leading to the detection of either intergenomic or intervariety polymorphism [12]. A number of SNPs have been identified in γ -gliadin genes [16] and explored using an allele-specific amplification approach. The cleaved amplified polymorphic sequence (dCAPS) method [17] was used to detect SNPs in the common wheat gene *Wx-D1* [18] and direct sequencing was used to detect SNPs in barley P450 cytochrome genes [19]. Single nucleotide polymorphism (SNP) in genes can result in a change of phenotype; thus, the detection of SNPs in plant coding sequences, with its potential for automation, can greatly accelerate molecular breeding [20].

The single nucleotide primer extension method has many acronyms [8]. It relies on the extension of a specific primer with labelled dideoxynucleotide(s) after

annealing to the PCR product. The primer ends one base before the mutation to be analysed, and the use of labelled ddNTPs with different, fluorescent dyes allows the simultaneous detection of multiple allelic variants including heterozygotes in a single reaction [21]. In this study, sequence data from the *T. tauschii* BAC library, obtained after screening with PSR567 probe [6], was used to design SNP markers for the detection of *Lr1* in common wheat. The purpose of this study was: 1) to identify point mutations in genomic sequences closely linked to the *Lr1* leaf rust resistance gene on the basis of information from the sequence database; and 2) to develop SNP markers using the primer extension method to aid breeders in the selection process targeting resistance genes pyramiding.

MATERIALS AND METHODS

Plant material

Studies were performed on 37 wheat cultivars, a set of 41 Thatcher near-isogenic lines of spring wheat and on a set of 21 doubled-haploid (DH) lines derived from 'Henika' (*Lr1*) x 'IPG-SW-14'. The Swiss cultivars were obtained from RAC, Station Federale de Recherches en Production Vegetale de Changins, Nyon, Switzerland. The British cultivars were supplied by the University of Reading, Reading, United Kingdom. The Czech and German cultivars originated from the Gene Bank of VURV, Research Institute of Crop Production, Prague-Ruzyně, the Czech Republic. The cultivars 'Agatha 235' and 'Teewon 2917' were supplied by Dr. W.J. Raupp, Department of Plant Pathology, Kansas State University, USA. The Mexican cultivars originated from the Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany. The seeds of the Polish cultivars were supplied by breeding companies in Szelejewo and Polanowice. The seeds of the Thatcher near-isogenic lines possessing resistance genes *Lr1-Lr44*, and the seeds of Thatcher were supplied by Dr. M. Czosz, Cereal Research Institute, Szeged, Hungary [22]. Seeds of accessions conferring *Lr47* were supplied by Prof. A. Łukaszewski, Department of Botany and Plant Sciences, University of California, Riverside, USA. A population of DH lines derived from cross Henika/IPG-SW-14 was developed by Dr. H. Wiśniewska, Institute of Plant Genetics Polish Academy of Science, Poznań, Poland. Inoculation tests were performed with *Lr1* virulent HK26-03 and *Lr1* avirulent 14 WxR isolates on 6 cultivars and 21 DH lines.

Identification of SNP

DNA sequences linked to the *Lr1* resistance gene at a distance of 0.04, i.e. AY123939, AY123940 and AY123941 related to PSR567 (AY123938) [6], were retrieved from the ENTREZ database [23]. Sequence AY123941 was used to search for similar sequences using the BLAST algorithm. Selected sequences were aligned with Clustal W using BioEdit [24]. Primers (Tab. 1) were manually selected out of those proposed by Primer 0.5 [25] and checked for secondary structure to avoid hairpin and multimer formation with Net Primer [26].

Tab. 1. Primers tested for SNP detection and the size of the expected products.

Primer name	Primer sequence 5' → 3'	Primer combination	Expected PCR size
Lr1_29F	gCTCCTATTTgCATCCTCCT	Lr1_29F/Lr1_154R	126
Lr1_43F	TCCTTgCCCCggCCCT	Lr1_43F/Lr1_154R	110
Lr1_98F	AgCTTACgAgAAgCAAgA	Lr1_98F/Lr1_154R	57
Lr1_197F	TTgCATCgCCTTCTCTCT	Lr1_197F/Lr1_275R	78
Lr1_154R	TAgTTTCTggAggAAgTgAgg	Lr1_29F/Lr1_275R	246
Lr1_275R	AggCCCTCCTgggCA		

PCR reaction

DNA was extracted using a previously described protocol [27]. The DNA concentration of samples was estimated on 1.5% Seakem agarose and adjusted to 10 ng/μL. PCR reactions were carried out in 20 μL volume containing 10 mM Tris-HCl, 50 mM HCl, 0.1% Triton X-100, 200 nM of each primer, 200 μM of each dNTP, 0.5 U of Taq (Finnzymes) and 25 ng of genomic DNA. PCR conditions were optimised for 1.5 mM and 2.5 mM concentrations of MgCl₂ at annealing temperatures optimal for respective primer pairs.

The following thermal profile was applied (GeneAmp 9700, Perkin Elmer): initial denaturation for 3 minutes at 94°C, 8 cycles of (45 s at 94°C, 45 s at 68°C (-1°C per cycle), 45 s at 72°C), 37 cycles of (45 s at 94°C, 45 s at 60°C, 45 s at 72°C), with a final elongation for 5 minutes at 72°C. 4 μL of the resulting products were resolved on 1.5% Seakem agarose.

SNP detection

5 μL of the PCR product were incubated with 1 U of shrimp alkaline phosphatase (SAP), and 0.1 U exonuclease I (ExoI) for 1 h at 37°C followed by a subsequent enzyme deactivation step for 15 min at 75°C. Primer extension (minisequencing) reactions were carried out in a volume of 10 μL, composed of 0.5 μL of premixed sequenase and fluorescently labelled dideoxynucleotides from a CEQ SNP Primer Extension Kit (Beckman Coulter), 1 μL of purified PCR product, 100 nM of primer and 0.6 X PCR buffer (6 mM TRIS-HCl, 0.9 mM MgCl₂, 30 mM KCl and 0.06% Triton X-100). The cycling profile applied was 30 cycles of 10 s at 96°C, 10 s at 50°C and 30 s at 72°C. 2.5 μL of labelled primer was cleaned up by incubation with 1 U of SAP for 30 minutes at 37°C followed by an inactivation step of 10 min at 80°C. 1 μL of sample was run on a CEQ™ 8000 Genetic Analysis System according to the manual.

RESULTS AND DISCUSSION

Design of SNP assay

The sequences similar to AY123941, which is linked at 0.04 cM to *Lr1* [6], were identified. BLAST was used to find additional sequences suitable for SNP design and to check for additional sequences that could interfere with the specificity of an assay. After initial alignment, the sequences AY123940 and

AY123939 were selected for nucleotide alignment in order to identify SNPs. No structural predictions were made. Taking into account the location of the sequences in proximity of *Lr1*, putative SNP detection in this region was likely be diagnostic for the *Lr1* gene. Multiple putative single nucleotide polymorphisms were identified in the 275 nt region, and 5 of them were selected for testing with the mini-sequencing (primer extension) method (Fig. 1). On the basis of the consensus sequence obtained from multiple alignment, six sites were selected for primer design, as listed in Tab. 1. Most of the primers (excluding Lr1_154) were designed to end next to the base where the mutation is expected.

	5	15	25	35	45	55	
AY123941	GGTGGATGAC	ATCTCTGGAT	TGCTTGCTGC	TCCTATTTCG	ATCCTCCTTG	CCCCGGCCCT	
AY123940	GGTGGATGAC	ATCTCTGGAT	TGCTTGTTGC	TCCTATTTCG	AACCTCCTCG	CCCCGGCCCT	
AY123939	TGTGGATAAC	AACTGTGGAT	TGCTTGTTGC	TCCTATTTCG	AACCTCCTCG	CCCCGGCCCT	
Consensus	-GTGGAT-AC	A-CT-TGGAT	TGCTTG-TGC	TCCTATTTCG	A- <u>CCTCCT</u> *G	CCCCGGCCCT	
	Lr1_29F Lr1_43F
	65	75	85	95	105	115	
AY123941	ACACACACTT	AAATTCTTGT	TTGATAAGAG	CATGGAAAAGC	TTCACGGAAG	AGCAAGACAA	
AY123940	CCGCATACCT	GAATTCAGGT	CTGATGGTAG	GACGGAAAAGC	TTCACGGAAG	AGCAAGATAA	
AY123939	CCACACACTT	GTATTCTGGA	TTGATGAAAC	GATGGAAAAGC	TTGACGGAAG	AGCAAGAGAA	
Consensus	*C-CA-ACTT	--ATTG--G-	-TGAT---A-	-A-GGAAAAGC	TT-ACGGAAG	<u>AGCAAGA</u> *AA	
	Lr1_98F
	125	135	145	155	165	175	
AY123941	AGCGCTGCAT	CTCCTCACCT	TCCTCCAGAA	ACTAAGTTTT	CACTCTTGCA	GGCGTTTGCA	
AY123940	AGCGCTGCAG	CTCCTCACCT	CCCTTGAGAA	ACTACATTTT	TTCATTTCG	AGGCTCTGCA	
AY123939	AGCGCTGCAG	CTCCTCACCT	CCCTCCAGAA	TCTAACATTT	TTCAGATGCA	GGGGTCTACA	
Consensus	AGCGCTGCA-	<u>CTCCTCACCT</u>	-CCT--AGAA	-CTA---TTT	--C---TGC-	-G-GT-T-CA	
	Lr1_154R
	185	195	205	215	225	235	
AY123941	GTCCCTTTCT	CAAGGGTTGC	ATCGCCTTTC	TTCTCTAAG	GAGTTACATG	TCCTTTGGTG	
AY123940	GTCCCTTTCT	CAAGGGTTAC	ATCGCCTTTC	TTCTCTCAAG	GAGTTACATG	TCAGGTACTG	
AY123939	GTCCCTTTCT	CAAGGGTTGC	ATCGCCTTTC	TTCTCTCAAG	GAGTTATGTG	TCCGTGGGTG	
Consensus	GTCCCTT*CT	CAAGGGTT-C	ATCGCCTTTC	<u>TTCTCT</u> *AAG	GAGTTA--TG	TC-----TG	
	Lr1_197F
	245	255	265	275			
AY123941	TCCAAAAATC	CGATCGATGC	CCAAGGAGGG	CCTCCCCTGTG			
AY123940	TCCAAATATC	CGATCGATGC	CCAAGGAGGG	CCTCCC----			
AY123939	TCTAAAAATC	CAATCGTTGC	CCAAGGAGGG	CCTCCC----			
Consensus	TC-AAA-ATC	C-ATCG*ATGC	CCAAGGAGGG	CCTCCC----			
							Lr1_275R

Fig. 1. Multiple alignment of sequences used for SNP assay design. Asterisks in consensus sequence mark targets for mutation detection with respective primers.

SNP assay optimisation

In the process of SNP assay development, PCR conditions were optimised to produce clear, single products. The primer combinations listed in Tab. 1 were tested for amplification using genomic DNA from the cultivars 'Henika', 'Frontana' and 'Thatcher'. Out of these, the combination of primers Lr1_29F and Lr1_275R with 2.5 mM of MgCl₂ was selected for subsequent primer purification and primer labelling as it generated a clear PCR product of expected size (~250 nt); besides this, it spanned the whole region of interest.

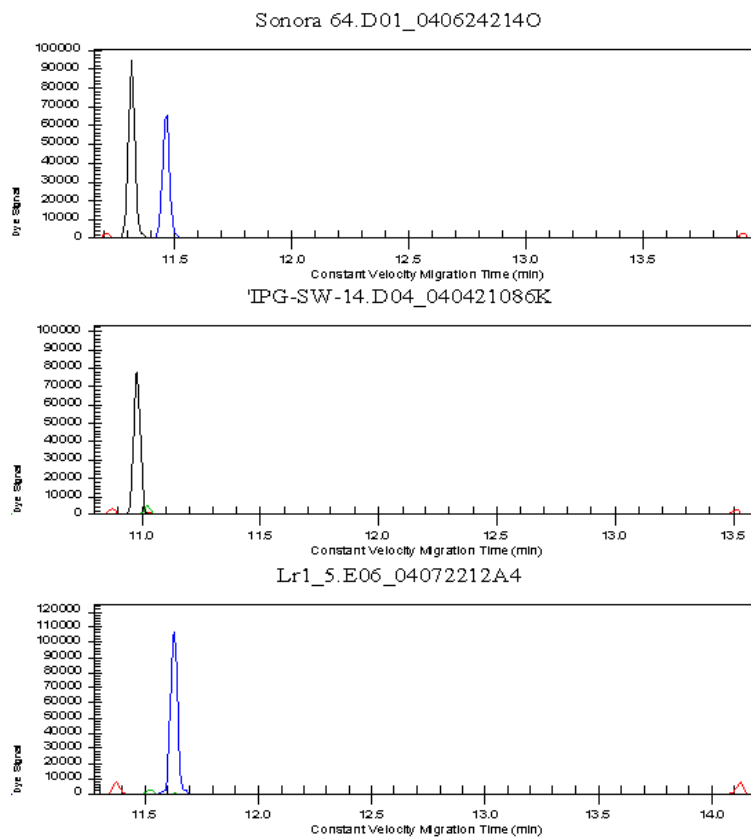


Fig. 2. Minisequencing of the PCR product (Lr1_29F+Lr1_275R) with the Lr1_98F primer for SNP detection.

PCR products obtained with Lr1_29F and Lr1_275R primers were subsequently tested for polymorphism in 5 putative point mutations. Six primers: Lr1_29F, Lr1_43F, Lr1_98F, Lr1_154R, Lr1_197F, and Lr1_275R were tested for SNPs on 11 genotypes: 'Thatcher_Lr1', 'Frontana', 'Henika', 'Santa', 'Thatcher', 'Agatha 235', 'Teewon 2917', 'Brigadier', 'Rialto', 'Kaukaz', 'Chinese Spring' and 'KS92WGRC15'. Verification of the putative SNPs with Lr1_29F, Lr1_43F, Lr1_197F and Lr1_275R was unsuccessful. These primers incorporated C, C, C, and T bases respectively. Although no polymorphic signals were found, the detected fragments confirmed the identity to sequence AY123940. Lr1_154R did not border the mutation and only an A signal was detected. Lr1_98F gave a polymorphic product on the genotypes differing by the presence/absence of the *Lr1* resistance gene including Thatcher and the isogenic line carrying the introduced *Lr1* gene ('Thatcher_Lr1'). The results obtained for the Lr1_98F primer suggest that more than one product was targeted for sequencing (Fig. 2).

SNP validation

In order to verify the usefulness of the SNP assay, additional analyses for the Lr1_98F primer were performed. The obtained SNP marker was tested on 32 wheat cultivars including 13 characterised for the presence/absence of the *Lr1* gene (Tab. 2, 3). The Lr1_98F primer detected four genotypes: T, C+T, C and 'null' in a set of tested *Triticum aestivum* varieties. Then, a population of 21 DH lines derived from the 'Henika' (*Lr1*) x 'IPG-SW-14' cross was tested. The C/T genotype was detected in 'Henika' and the C allele was found in IPG-SW-14.

Tab. 2. SNP typing on germplasms with recognized characteristics for *Lr1*.

Cultivar	Country of origin	Postulated <i>Lr1</i> genes	
		as a reaction type	Lr1_98F
Henika	PL	+	C/T
Nuri F70	MEX	+	C/T
Noroeste 66	MEX	+	C/T
Santa	PL	+	C/T
Sonora 64	MEX	+	C/T
Sonoita F81	MEX	+	T
Tobari	MEX	+	C/T
Tonichi S81	MEX	+	C/T
Vlada	CZ	+	C/T
Yecora Rojo Lr47	MEX	+	C/T
Agatha 235	USA	-	'null'
Brigadier	GB	-	C
Chinese Spring	CHN	-	C
Frontana	BR	-	C
KS92WGRC15	USA	-	C
Olymp	PL	-	C
Teewon 2917	USA	-	C

The segregation of T in the progeny was 6 C/T:15 C. SNP markers are not expected to behave in a dominant manner, as their ability to detect the heterozygous state is well known. The presence of the *Lr1* gene was supposed to be linked with the T allele, while the C allele was considered not to be associated with *Lr1*. This situation can be explained by the presence of three sites of primer binding in minisequencing. The BAC clone 6O14 from *T. tauschii* contained two sequences hybridizing with the PSR567 probe [6]. The co-detection of C along with T may also be due to the occurrence of the investigated sequences at several loci in the wheat genome. This was shown by hybridization of the probe PSR567 to loci at 5BL, 5DL and 7BS [6].

Tab. 3. SNP typing on germplasms with unknown characteristics for *Lr1*.

Cultivar	Country of origin	Lr1_98F	Cultivar	Country of origin	Lr1_98F
Alka	CZ	C	Flair	D	'null'
Alkora	PL	C	Hera	PL	C
Almari	PL	C	Husar	GB	C
Banti	PL	C	Igna	PL	C
Bill	DK	C	Ismena	PL	C
Caphorn	FR	C	Jasna	PL	C
Clever	GB	C	Pegasso	I	C
Corsaire	FR	C	Renan	FR	C
Danis	CH	C	Terza	CH	C
Eta	PL	C	Titlis	CH	C

The variety 'Thatcher' and 41 near isogenic lines with backcrossed genes (*Lr1*, *Lr2a*, *Lr2b*, *Lr2c*, *Lr3*, *Lr3ka*, *Lr3bg*, *Lr9*, *Lr10*, *Lr11*, *Lr12*, *Lr13*, *Lr14a*, *Lr14b*, *Lr15*, *Lr16*, *Lr17*, *Lr18*, *Lr19*, *Lr20*, *Lr21*, *Lr22*, *Lr23*, *Lr24*, *Lr25*, *Lr26*, *Lr28*, *Lr29*, *Lr30*, *Lr32*, *Lr33*, *Lr34*, *Lr35*, *Lr37*, *Lr38a*, *Lr38b*, *Lr44a*, *Lr44b*, *LrW*, *LrB*, *LrBb*) were tested along with the Pavon *Lr47* line carrying the gene *Lr47*. The diagnostic T allele was found in the line with added *Lr1* (T) and the *Lr3ka* (C/T) resistance genes. 'Thatcher' and the other isogenic lines tested showed the presence of the 'null' allele. The *Lr3* complex locus is located on wheat chromosome 6BL [1]. It remains to be verified whether the *Lr1* gene was introduced into the Thatcher_ *Lr3ka* line on the base of a similar response to leaf rust isolates or whether this SNP also detects *Lr3ka*.

The inoculation tests performed on DH lines, parents and selected cultivars confirmed the results of SNP typing. An additional *Lr* gene was found in the DH population, making verification of *Lr1* presence unambiguous in only 6 lines. In one DH-line (DH40), the *Lr1* specific allele was not detected indicating that resistance to both isolates tested is presumably caused by the presence of additional *Lr* genes.

SNP markers have the potential to be used in an automated process supporting marker-assisted selection in breeding programs. A semi-automated system for sequence analysis and SNP storage has been proposed [28]. The development of liquid-handling robots allows the acceleration of many manipulations on large populations. Particularly in the case of marker-assisted plant breeding, a special, pressing need exists for robust, high-throughput and cost-efficient SNP scoring methods. At present, the requirement of a PCR amplification step prior to SNP genotyping is the main factor delimiting the throughput of the assay. Several strategies can be applied to reduce the costs of analyses. In the melting curve SNP (McSNP) method, reagent costs are less than 0.10 USD per genotype [29]. This method exploits the detection of differences in the melting temperatures of fragments after digestion with restriction endonucleases [30]. SNPs fitting to these methods can easily be identified by converting known STS-RFLP markers, or the restriction site can be artificially introduced into PCR products by primer

mismatch. Analysis of the DNA melting curve is suitable for scoring diallelic variants [30]. In our study, we identified a triallelic SNP, and all the putative biallelic mutations tested were not informative.

Pyrosequencing can be the method of choice when working on pools of DNA, as the intensity of signals facilitates the estimation of allele frequencies [31]. Direct detection of SNP with allele-specific amplification was possible in case of the abundant group of γ -gliadin genes with extremely low primer concentrations in PCR mixes (5pmol) to enhance accuracy [16]. Another marker system for direct SNP detection is the ligase chain reaction (LCR), which uses primers with attached overhangs and can be considered a relatively cheap method. It was proven to be useful for the detection of genomic DNA starting from 0.1 ng of DNA in case of *Phytophthora* followed by separation on acrylamide and EtBr staining [32]. It is interesting as it works on genomic DNA and not on PCR products, and allows the detection of mutation without prior PCR reaction and the accompanying purification steps.

However, for wheat, minisequencing is promising, as our results demonstrate. The PCR required prior to SNP detection allows the enhancement of the specificity of the assay, thus reducing the influence of the complex genome. In case of the primer extension technique, primers can be designed to allow 45-plexing of the minisequencing reaction [21]. The growing number of wheat SNP markers will open the possibility to introduce multiplexed assays targeting loci of special interest in the breeding process by marker-assisted selection.

Acknowledgements. This study was supported by the State Committee for Scientific Research in Poland, project No. PBZ/KBN/029/P06/2000.

REFERENCES

1. McIntosh, R.A., Hart, G.E., Devos, K.M., Gale, M.D. and Rogers, W.J. Catalogue of gene symbols for wheat. Proc. 9th Int. Wheat Genetic Symp. Saskatoon, Canada. 2-7 August. Univ. Saskatchewan. (2003) (<http://wheat.pw.usda.gov/ggpages/wgc/98/>).
2. Dyck, P.L. and Samborski, D.J. Genetics of resistance to leaf rust in common wheat varieties Webster, Loros, Brevit, Carina, Malakoff and Centenario. **Can. J. Genet. Cytol.** 10 (1968) 7-17.
3. McIntosh, R.A., Baker, E.P. and Driscoll, C.J. Cytogenetical studies in wheat. I. Monosomic analysis of leaf rust resistance in the cultivars Uruguay and Transfer. **Aust. J. Biol. Sci.** 18 (1965) 971-977.
4. Feuillet, C., Messmer, M., Schachermayr, G. and Keller, B. Genetic and physical characterization of the LR1 leaf rust resistance locus in wheat (*Triticum aestivum* L.) **Mol. Gen. Genet.** 248 (1995) 553-562.
5. Schachermayr, G., Feuillet, C. and Keller, B. Molecular markers for the detection of wheat leaf rust resistance gene *Lr10* in diverse genetic background. **Mol. Breed.** 3 (1997) 65-74.

6. Ling, H.-Q., Zhu, Y. and Keller, B. High-resolution mapping of the leaf rust disease resistance gene *Lr1* in wheat and characterization of BAC clones from the *Lr1* locus. **Theor. Appl. Genet.** 106 (2003) 875-882.
7. Syvänen, A.C. From gel to chips: "minisequencing" primer extension for analysis of point mutations and single nucleotide polymorphisms. **Hum. Mutat.** 13 (1999) 1-10.
8. Syvänen, A.C. Accessing genetic variation: genotyping single nucleotide polymorphisms. **Nature Rev.** 2 (2001) 930-942.
9. Mohler V. SNP genotype determination in hexaploid wheat. In: **Microscopic fungi – host resistance genes, genetics and molecular research.** (Chelkowski, J. and Stepień, Ł. Eds.), 2004, 53-58.
10. Ching, A. and Rafalski, A. Rapid genetic mapping of ESTs using SNP pyrosequencing and indel analysis. **Cell. Mol. Biol. Lett.** 7 (2002) 803-810.
11. Somers, D.J., Kirkpatrick, R., Moniwa, M. and Walsh, A. Mining single-nucleotide polymorphisms from hexaploid wheat ESTs. **Genome** 49 (2003) 431-437.
12. Ablett, G.A., Shapter, F., Bowen, S. and Henry, R.J. SNP discovery in microsatellites and ESTs in hexaploid wheat using pyrosequencing. **Proc. 10th Int. Wheat Genetics Symposium**, Paestrum, Italy, 2003, 919-921.
13. Mochida, K., Kawaura, K. and Ogihara, Y. SNP genotyping of hexaploid wheat by the "Allele-specific pyrosequencing". **Proc. 10th Int. Wheat Genetics Symposium**, 1-6 Sept. 2003, Paestrum, Italy, (2003) 1003-1005.
14. Rafalski, A.J. Novel genetic mapping tools in plants: SNPs and LD-based approaches. **Plant Sci.** 162 (2002) 329-333.
15. Pacey-Miller, T. and Henry, R. Single-nucleotide polymorphism detection in plants using a single-stranded pyrosequencing protocol with universal biotinylated primer. **Anal. Biochem.** 317 (2003) 165-170.
16. Zhang, W., Gianibelli, M.C., Ma, W., Rampling, L. and Gale, K.R. Identification of SNPs and development of allele-specific PCR markers for γ -gliadin alleles in *Triticum aestivum*. **Theor. Appl. Genet.** 107 (2003) 130-138.
17. Neff, M.M., Neff, J.D., Chory, J. and Pepper, A.E. dCAPS, a simple technique for genetic analysis of single nucleotide polymorphisms: experimental applications in *Arabidopsis thaliana* genetics. **Plant J.** 14 (1998) 387-392.
18. Yanagisawa, T., Kiribuchi-Otobe, C., Hirano, H., Suzuki, Y. and Fujita, M. Detection of single nucleotide polymorphism (SNP) controlling the waxy character in wheat by using a derived cleaved amplified polymorphic sequence (dCAPS) marker. **Theor. Appl. Genet.** 107 (2003) 84-88.
19. Bundock, P.C., Christopher, J.T., Eggler, P., Ablett, G., Henry, R.J. and Holton, T.A. Single nucleotide polymorphisms in cytochrome P450 genes from barley. **Theor. Appl. Genet.** 106 (2003) 676-682.
20. Wang, Z. and Moulton, J. SNPs, protein structure, and disease. **Hum. Mutat.** 17 (2001) 263-270.

21. Kaderali, L., Deshpande, A., Nolan, J.P. and White, P.S. Primer-design for multiplexed genotyping. **Nucleic Acids Res.** 31 (2003) 1796-1802.
22. Chełkowski, J., Golka, L. and Stępień, Ł. Application of STS markers for leaf rust resistance genes in near-isogenic lines of spring wheat cv. Thatcher. **J. Appl. Genet.** 44 (2003) 323-338.
23. ENTREZ, URL: <http://www.ncbi.nlm.nih.gov/entrez/>
24. Hall, T.A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. **Nucleic Acids Symp. Ser.** 41 (1999) 95-98.
25. Daly, M.J., Lincoln, S.E. and Lander, E.S. "PRIMER", unpublished software Whitehead Institute/MIT Center for Genome Research. (1991) <http://www-genome.wi.mit.edu/ftp/pub/software/primer.0.5>
26. NetPrimer, URL: <http://www.premierbiosoft.com/netprimer/>
27. Stępień, Ł. Holubec, V. and Chełkowski, J. Resistance genes in wild accessions of Triticeae – inoculation test and STS marker analyses. **J. Appl. Genet.** 43 (2002) 423-435.
28. Lehnert, V., Holzwarth, J., Ott, M., Thompson, A., Demmak, S. and Foerzel, D. A semi-automated system for analysis and storage of SNPs. **Hum. Mutat.** 17 (2001) 243-254.
29. Shirver, M.D. and Akey, J.M. SNP 2000: Third international meeting on single nucleotide polymorphism and complex genome analysis. Melting curve SNP (McSNP) genotyping: a simple gel free low-cost approach to SNP genotyping and DNA fragment analysis. **Hum. Mutat.** 17 (2001) 317-347.
30. Ye, J., Parra, J.E., Sosnoski, D.M., Hiester, K., Underhill, P.A. and Shriver, M.D. Melting curve SNP (McSNP) genotyping: a useful approach for diallelic genotyping in forensic science. **J. Forensic. Sci.** 47 (2002) 593-600.
31. Sylvan, A. SNP 2000: Third international meeting on single nucleotide polymorphism and complex genome analysis. Accurate allele frequency estimation of SNPs using pyrosequencing. **Hum. Mutat.** 17 (2001) 317-347.
32. Tooley, P.W., Hatziloukas, E., Scott, D.L. and Carras, M.M. Use of ligase chain reaction for enhanced detection of *Phytophthora infestans*. **Can. J. Plant. Pathol.** 24 (2002) 294-301.