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EcoTILLING for the identification of allelic variation within the powdery mildew resistance genes *mlo* and *Mla* of barley

Mejlhede, N.[1)], Kyjovska, Z.[2)], Backes, G.[1)], Burhenne, K.[3)], Rasmussen S. K.[1)], and Jahoor, A.[1)]

[1)] *Department of Agricultural Sciences, The Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Copenhagen, Denmark*

[2)]*Present address: Masaryk University, Department of Genetics and Molecular Biology, Faculty of Science, Kotláøska 2, 60000 Brno, Czech Republic*

[3)] *Present address: Novozymes A/S, Hallas Alle 1 building AXS.36, DK-4400 Kalundborg, Denmark*

Corresponding author:

Jahoor, A.
E-mail: aha@kvl.dk
Phone: +45 35283466
Fax: +45 35283468

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ABSTRACT

In this investigation we describe the successful implementation of a CEL I-based mutation detection technique for discovery and detection of DNA polymorphism in the genes mlo and Mla of barley. The technique is called EcoTILLING, which is a high throughput method to detect and discover new point mutations and small insertions/deletions in DNA. We demonstrate that the method not only reveals polymorphism between different alleles, but also can be used as a powerful genetic marker. The genes mlo and Mla are involved in the defence of barley to the fungal pathogen powdery mildew. The powdery mildew resistance gene mlo is a single copy gene, whereas multiple alleles exist at the Mla locus. With EcoTILLING we were able to identify point mutations and deletions in each of the 11 mlo mutants tested. For Mla we tested 25 natural barley variants, and although the identification was complex due to the presence of highly similar paralogs of Mla, we were able to identify most of the recently identified alleles from Hordeum vulgare ssp. spontaneum. This method offers the possibility to combine different mlo alleles with different Mla alleles from wild barley to obtain cultivars with more durable resistance.

INTRODUCTION

EcoTILLING is a high throughput method to detect and discover new point mutations and small insertions/deletions in DNA (Comai et al. 2004). The method is gel-based and thereby low cost. EcoTILLING is a variant of TILLING. TILLING is a high throughput non-transgenic strategy for providing allelic series of mutations, including knock-outs (McCallum et al. 2000). It permits the identification of mutations in target genes without the implementation of genetically modified organisms that cause public concern in Europe. The method is therefore of great interest in commercial agriculture.

The wild type *Mlo* gene encodes a protein, which is probably involved in regulating a cell wall repair process. Its malfunction results in excessive papilla growth and a high level of resistance to powdery mildew. A number of *mlo* mutants have been reported, of these at least 32 are of proven independent mutational origin and most of them have been sequenced. They are assigned resistance gene symbols *mlo*1 to *mlo*32 (Jørgensen 1994, Büschges et al. 1997, Piffanelli et al. 2002, Molina-Cano et al. 2003). Of these, most have been induced by chemical mutagens, five have been induced with radiation, and one (*mlo*11) is naturally occurring in Ethiopian landraces (Piffanelli et al. 2004). The known inactivated *mlo* alleles are either characterized by a single point mutation or by deletion of a few nucleotides. Currently, the only way to identify alleles in the *mlo* gene is either by pedigree or by sequencing of the gene.

The *Mla* locus is of great interest because of the diversity of resistant phenotypes that are conferred by different *Mla* resistance specificities. These phenotypes can range from near immunity, associated with a rapid hypersensitive response and early growth arrest of the powdery mildew, to a late response allowing the development of some fungal mycelium

(Boyd et al. 1995). The *Mla* region contains multiple classes of genes associated with plant defence responses and genetic variants of the *Mla* locus are found in cultivars worldwide.

At least 20 very effective alleles of the *Mla* locus have been identified in a wild barley collected in Israel (Jahoor and Fischbeck 1987, Jahoor and Fischbeck 1993, Kintzios et al. 1995). Currently *Mla* alleles are mostly determined by resistance tests with a set of powdery mildew isolates. However, the isolates collected in Europe do not possess the corresponding virulence genes to detect the alleles of the *Mla* locus from wild barley. Therefore, isolates sampled from wild barley in Israel have to be employed for this purpose.

In this study we demonstrate that point mutations in the *mlo* gene and the natural variation at the *Mla* locus of barley can easily be detected by EcoTILLING.

MATERIALS AND METHODS

Plant material

The plant lines used in this work consisted of 11 barley *mlo* mutant lines, the cultivar 'Ingrid' (*Mlo* WT) and 25 barley lines including cultivars from Europe, near isogenic lines in Pallas background (Kølster et al. 1986), and derivatives of wild barley containing known *Mla* alleles (Jahoor and Fischbeck 1987, Jahoor and Fischbeck 1993, Kintzios et al. 1995).

DNA isolation

One to two leaves of 8 days old seedlings were freeze-dried, ball-milled with two 3-mm steel-balls in a ball-mill (Retsch) and plant DNA was isolated in a 2 ml reaction tube according to the CTAB-protocol (Saghai-Maroof et al. 1984).

CEL I purification

CEL I was purified after a modified protocol from Oleykowski *et al.* (1998) using an ÄKTAexplorer 10S chromatography system from Amersham Pharmacia Biotech. All steps were performed at 4°C.

Step 1: Celery stalks (6.3 kg fresh from the grocery shop) were juiced in a blender and adjusted to give the composition of buffer A (0.1 M Tris pH 7.7 containing 0.1 mM PMSF). The crude extract was filtered through a nylon mesh and centrifuged at 10,000 *g* for 30 min. The supernatant was gently stirred and solid $(NH_4)_2SO_4$ was slowly added to a final concentration of 25% saturation. After 1 h, the suspension was centrifuged at 20,000 *g* for 60 min. The supernatant was adjusted to 80% $(NH_4)_2SO_4$ saturation and stirred for one hour before centrifugation at 20,000 *g* for 60 min. The pellet was resuspended in 400 ml buffer B (buffer A containing 0.5 M KCl) and thoroughly dialyzed against buffer B.

Step 2: Con A Sepharose 4B (60 ml) from Amersham Biosciences was equilibrated in buffer B. The dialyzed extract was gently stirred for 2 h with 50 ml Con A resin and then packed in a 2.6 diameter column prepacked with 10 ml Con A resin. The resin was washed in 600 ml

buffer B before bound CEL I was batch eluted in 250 ml buffer C (buffer B containing 0.01% Triton X-100 and 0.3 M methyl-α-D-mannopyranoside) at a flow rate of 3 ml/min. Eluted protein was dialyzed against buffer D (50 mM Tris pH 8.0 containing 5 mM methyl-α-D-mannopyranoside, 0.01% Triton X-100 and 25 µM PMSF).

Step 3: The enzyme extract was loaded onto a HiPrep 16/10 Q XL column equilibrated in buffer D. The column was washed in 400 ml buffer D before a linear gradient (0-100% in 400 ml) of buffer E (buffer D containing 0.5 M KCl) was applied at a flow rate of 5 ml/min. Fractions of 4 ml were collected and assayed for CEL I activity. The most active CEL I fractions were pooled and dialyzed against buffer D.

Step 4: The extract was loaded onto a Mono Q (1 ml) column equilibrated in buffer D. The column was washed in 20 ml buffer D before a linear gradient (0-100% in 50 ml) of buffer E was applied at a flow rate of 1 ml/min. Fractions of 1 ml were collected. The pUC19 RF-I nicking assay described by Yang *et al.* (2000) was used to quantify CEL I throughout purification.

PCR primers

Primers and 5' fluorescent-labelled primers were obtained from Applied Biosystems. Fluorescent-labelled primers contained 5'-labelled VIC-dye (green) or 5'-labelled 6-FAM dye (blue). The primer design was based on the published sequences of *Mlo* (accession no Y14573) and *Mla* (accession no AY009938) with melting temperatures around 60ºC. The software 'Primer3' (Rozen and Skaletsky 1988) was used in the primer design. Primer mixes for PCR consisted of 4 primers: labelled and unlabelled forward primers together with labelled and unlabelled reverse primers in the ratio 2:3 (labelled: unlabelled), for a final concentration of 0.5 µM in the PCR.

PCR amplification

PCR was performed in 20 µl volumes using 20 ng of extracted DNA (from one or several plants), 1x Taq polymerase buffer (Promega), 0.2mM dNTPs, 2.5mM $MgCl_2$, 0.5 µM primer mix and 0.5 unit Taq Polymerase (Promega).

Amplifications were performed on a GeneAmp PCR System 2700 thermal cycler (PE Applied Biosystem) as follows: 95°C for 2 min; eight cycles of touchdown PCR (94°C for 30 s, 68°C for 30 s decrementing 1°C per cycle, 72°C for 1 min); 45 cycles of: (94°C for 30 s, 60°C for 30 s, 72°C for 1 min), 72°C for 5 min; denaturation at 99°C for 10 min; and slow renaturation with 70 cycles of 30 s at 70°C to 49°C, decrementing 0.3°C per cycle. The last steps in the program inactivate the polymerase, denature the DNA strands and slowly reanneal the strands for the heteroduplex formation. Cycling was followed by CEL I digestion, cleanup, gel electrophoresis, and scanning.

Table 1: PCR primer used for EcoTILLING of *mlo* and *Mla*.

Name	***Oligonucleotide sequence (5´- 3´)***
mloA-F	CGTGTGCGTACCTGGTAGAG
mloA-F-VIC	VIC-CGTGTGCGTACCTGGTAGAG
mloA-R	CAAGCCAAGACGACAATCAG
mloA-R-FAM	6-FAM-CAAGCCAAGACGACAATCAG
mloB-F	CTGATTGTCGTCTTGGCTTG
mloB-F-VIC	VIC -CTGATTGTCGTCTTGGCTTG
mloB-R	CTGACTCCATACGCCAAACA
mloB-R-FAM	6-FAM -CTGACTCCATACGCCAAACA
mloC-F	TGTTTGGCGTATGGAGTCAG
mloC-F-VIC	VIC-TGTTTGGCGTATGGAGTCAG
mloC-R	AGAAACCGGAGAGGAGAAGG
mloC-R-FAM	6-FAM -AGAAACCGGAGAGGAGAAGG
mloD-F	CCTCACCCTCTTCCTTGACA
mloD-F-VIC	VIC-CCTCACCCTCTTCCTTGACA
mloD-R	CGTCAGAGCAGTTCATCAGC
mloD-R-FAM	6-FAM -CGTCAGAGCAGTTCATCAGC
mloE-F	CCACCGATGAACTTGTCAGT
mloE-F-VIC	VIC-CCACCGATGAACTTGTCAGT
mloE-R	GAGAGGGGTTTTGTTTGTGC
mloE-R-FAM	6-FAM -GAGAGGGGTTTTGTTTGTGC
Mla1-3F	AGCAGCTCGACAGCCAAGACAA
Mla1-3FFAM	6-FAM -AGCAGCTCGACAGCCAAGACAA
Mla1-3R	CCCAACCCTCCAAATCCAACAA
Mla1-3RVIC	VIC-CCCAACCCTCCAAATCCAACAA

6-FAM and VIC are 5' end labelled fluorescence dyes.

CEL I digestion

The assay was carried out as described by Colbert *et al.* (2001). For digestion of 10 µl PCR products in 96-well plates, 20 µl of a solution containing 10 mM HEPES (pH 7.5), 10 mM $MgSO_4$, 0.002% (w/v) Triton X-100, 0.2 µg ml^{-1} of bovine serum albumin, and 1/300 dilution of CEL I was added, and the plate was incubated at 45°C for 60 min. Reactions were stopped by addition of 5 µl 0.2 M EDTA (pH 8) on ice and the mixture pipetted into wells of a Millipore multiscreen system 96 well filtration plate containing Sephadex G-50 fine (Amersham Biosciences). The eluates were mixed with 5 µl of formamide loading solution containing ROX-1000 internal lane standard (200 µg ml^{-1} bromophenol blue and 1/16 dilution of GeneScan-1000 ROX size standard (Applied Biosystems) in deionised formamide). The sample volume was reduced to 5 µl by incubation at 85°C for 45 minutes and then stored on ice.

Table 2: Description of *mlo*-alleles used in this study

Allele	***variety (mutagen)***	***Mutational event***[1]	***Effect on MLO protein***	***Primer mix***[2]
mlo1	Haisa (X-rays)	T484→A	Trp162→Arg	mloB
mlo3	Malteria Heda (γ-rays)	Deletion of two nucleotides 1188-1189	Frameshift after Phe395	mloE
mlo4	Foma (X-rays)	Deletion of 11 nucleotides 478-488	Frameshift after Trp159	mloB
mlo5	Carlsberg II (EMS)	G3→A	Met1→Ile	mloA
mlo7	Carlsberg II (EMS)	G677→A	Gly226→Asp	mloC
mlo8	Carlsberg II (EMS)	A1→G	Met1→Val	mloA
mlo9	Diamant (EMS)	C28→T	Arg10→Trp	mloA
mlo10	Foma (γ-rays)	Deletion of 6 nucleotides 543-548	Missing Phe182 and Thr183	mloB
mlo12	Elgina (Nitrosomethylurea)	C1396→A	Phe240→Leu	mloC
mlo13	Plena (EMS)	T89→A	Val30→Glu	mloA
mlo17	Plena (EMS)	C92→T	Ser31→Phe	mloA

[1]Numbers of nucleotides after the translational start site.

[2]Primer mix refers to primers listed in Table 1.

EMS = ethylmethane sulfonate.

References: (Büschges et al. 1997) and (Piffanelli et al. 2002).

Detection of DNA fragments

The samples were heated for 96°C for 3 min, loaded onto a denaturing 36 cm well-to-read 5% polyacrylamide gel. We chose to load only 48 samples on a 96-well gel, since the lanes are very close and strong signals in one lane can sometimes be detected in the neighbouring lanes. The gel was run and analyzed on an ABI PRISM 377 DNA Sequencer using GeneScan 3.1 Software (Perkin Elmer). Results can be displayed as a gel image or as an electropherogram that displays the peak profile of each lane of the gel image.

RESULTS

Design of primers for *mlo*

Selection of primers was based on the *Mlo* sequence from *Hordeum vulgare* ssp. *vulgare* cultivar "Ingrid" which was available through the NCBI GenBank®. The *Mlo* gene consists of 3163 base pairs including a 5'-UTR (224 base pairs), a coding sequence interrupted by 11 introns, and a 3'-UTR (100 base pairs). Figure 1 shows the overall gene structure. The 12 exons vary in length from 41 base pairs to 396 base pairs. In order to cover the whole coding sequence five primer pairs were designed. The sizes of the five corresponding PCR products were 599, 624, 566, 574 and 837 base pairs. Table 2 summarizes the information on *mlo* mutants used in this study.

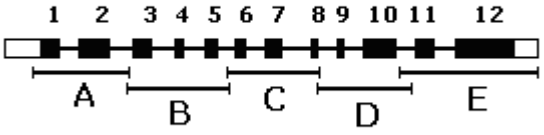


Figure 1: The *Mlo* gene. Exons are shown as boxes, black parts are the coding sequence and white parts are the 5'UTR and 3'UTR, respectively. Introns are shown as thick black lines. Primer design is illustrated below the gene, where each letter represents the region covered by the respective primer pairs A, B, C, D and E. Sizes of PCR products were 599, 624, 566, 574 and 837 base pairs, respectively. Exon numbers are indicated above the gene.

The *mlo* gene is 3163 base pairs long.

CEL I-based mutation detection of *mlo*

We tested DNA from the mutants *mlo*1, *mlo*3, *mlo*4, *mlo*5, *mlo*7, *mlo*8, *mlo*9, *mlo*10, *mlo*12, *mlo*13 and *mlo*17. Mutants *mlo*5, *mlo*8, *mlo*9 , *mlo*13 and *mlo*17 have single point mutations in exon 1 and we confirmed these point mutations using primer pair A. Mutant *mlo*1 has a single point mutation in exon 4, *mlo*4 has a 11 base pairs deletion in exon 4 and *mlo*10 has a 6 base pairs deletion in exon 5 and these three mutants were tested with primer pair B. Mutant *mlo*7 has a single point mutation in exon 6 and *mlo*12 has a single point mutations in exon 7 and these two mutants were tested with primer pair C. Mutant *mlo*3 has a 2 base pairs deletion in exon 11 and was tested with primer pair E.

As an example, Figure 2 shows a gel image of mutation detection analysis of *mlo*3 and WT using primer pair E. DNA from *Hordeum vulgare* ssp. *vulgare* cultivar "Ingrid" was mixed in a ratio of 1:1 with DNA from *mlo*3. The PCR and CEL I digestion were carried out as described in material and methods. Mutant *mlo*3 has a deletion of two nucleotides in the *mlo* gene compared to WT, and this deletion will result in a heteroduplex formation with a 2 nucleotide DNA loop. The PCR product was then subjected to CEL I endonuclease treatment. CEL I enzyme cuts one of the two DNA strands 3' of a mismatch, which can be a single point mutation or in this case a loop. When the DNA is denatured, the result is fluorescence labelled truncated fragments with either VIC (green), or 6-FAM (blue), which can be detected by an

ABI automated DNA sequencer. With the primer mix E used for *mlo*3 and WT, the expected lengths of the truncated fragments are 198 nucleotides VIC (green) and 641 nucleotides 6-FAM (blue). Fragments detected by the ABI sequencer were 199 nucleotides VIC (green) and 647 nucleotides 6-FAM (blue) which is in agreement with the expected fragment sizes. The same procedure was repeated for the other *mlo* mutants.

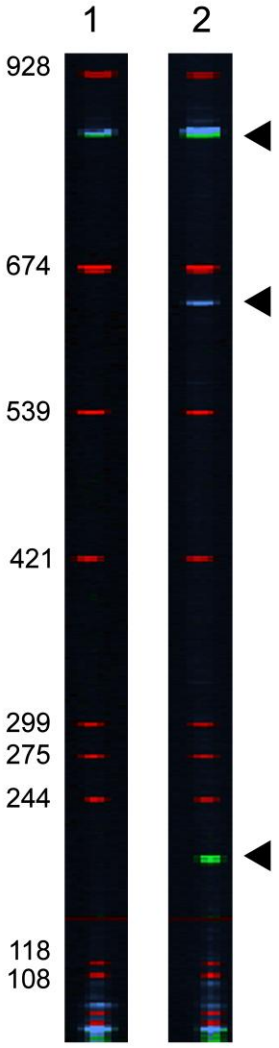


Figure 2: A gel image of mutation detection analyses on an ABI automated DNA sequencer running the GeneScan program. The substrate is an 835-837 base pairs heteroduplexed PCR product using DNA from WT and mutant *mlo*3 and primer mix E, which covers exon 11 and exon 12 of *mlo*. It is labelled at the 5′ terminal with VIC (green) on one strand and 6-FAM (blue) on the othmer. The substrate was incubated with CEL I enzyme for 60 min. at 45°C and then analyzed. In lane 1 the control reaction is shown where PCR is done using DNA from WT only. In lane 2 the reaction is shown when DNA from WT and *mlo*3 is mixed. Mutant *mlo*3 contains a 2 nucleotide deletion compared to WT. The blue fragment at approximately 641 nucleotides corresponds to CEL I mismatch-specific cutting on the 6-FAM-labelled strand, and the green peak at approximately 198 nucleotides corresponds to the mismatch-specific cutting on the VIC-labelled strand. The strong fragments in green and blue in each lane correspond to the full-size uncut PCR fragments of approximately 835-837 nucleotides in length. The sizes of the red standard (ROX-1000) are shown on the left.

Table 3: Cel I fragment sizes with labelled primers 6-FAM and VIC observed for the different *mlo* mutants by EcoTILLING.

WT + mutant	***Expected size[a]***		***Observed size[a]***		***Primer mix****
	VIC	***6-FAM***	***VIC***	***6-FAM***	
mlo1	313	312	315	312	mloB
mlo3	198	641	199	647	mloE
mlo4	317	318	318	319	mloB
mlo5	73	527	73[b]	526	mloA
mlo7	123	444	124	444	mloC
mlo8	71	529	71[b]	528	mloA
mlo9	98	502	99[b]	502	mloA
mlo10	499	131	500	128	mloB
mlo12	282	285	283	285	mloC
mlo13	159	441	160	442	mloA
mlo17	162	438	163	437	mloA

[a] Values are in nucleotides. [b] We could observe a fragment of this size, but in this area of the gel many fragments are present. *Primer mix refers to primers listed in Table 1.

In the lower area of the gel, many fragments are abundant. Therefore analysis of the gel in this area is complicated. The noisy signal in the area 0-100 nucleotides is due to the presence of excess fluorescence primers and degraded PCR products with attached fluorescence dyes.

The *mlo5*, *mlo8* and *mlo9* contained mutations close to the primer site; 73, 71 and 98 nucleotides, respectively, and the mutations were therefore confirmed primarily by the presence of the corresponding blue fragment of sizes 526, 528 and 502 nucleotides, respectively. A way to avoid the problem is to locate a primer more distant to the mutation site.

For all of the *mlo* mutants, the sizes of the fragments generated by EcoTILLING were in agreement with the expected sizes calculated from the sequence information given from the database within a few nucleotides.

Design of primers for *Mla*

Selection of primers for the *Mla* gene was based on an alignment of the coding sequences of *Mla1*, *Mla6*, *Mla12* and *Mla13* from *Hordeum vulgare* ssp. *vulgare* available through the NCBI GenBank ®.

The overall gene structure of *Mla1* is shown in Figure 3. The gene consists of 4665 base pairs including a 5´-UTR (229 base pairs) interrupted by an intron, a coding sequence (2877 base pairs) interrupted by two introns and a 3´-UTR (234 base pairs).

Figure 3: The *Mla1* gene. Exons are shown as boxes, black parts are the coding sequence and white parts are the 5'UTR and 3'UTR respectively. Introns are shown as thick black lines. Primer design is illustrated below the gene. The region covered by the primer pair is 451 base pairs. The gene is 4665 base pairs in size.

The coding sequence of *Mla1* is shown in Figure 4, where shaded nucleotides indicate polymorphic sites for *Mla6*, *Mla12* and *Mla13*. The 3' end shows a high level of polymorphism between alleles and is not suitable for EcoTILLING. Therefore, we chose the 5' end for the primer design as shown in Figure 4. The size of the corresponding PCR product is 451 base pairs.

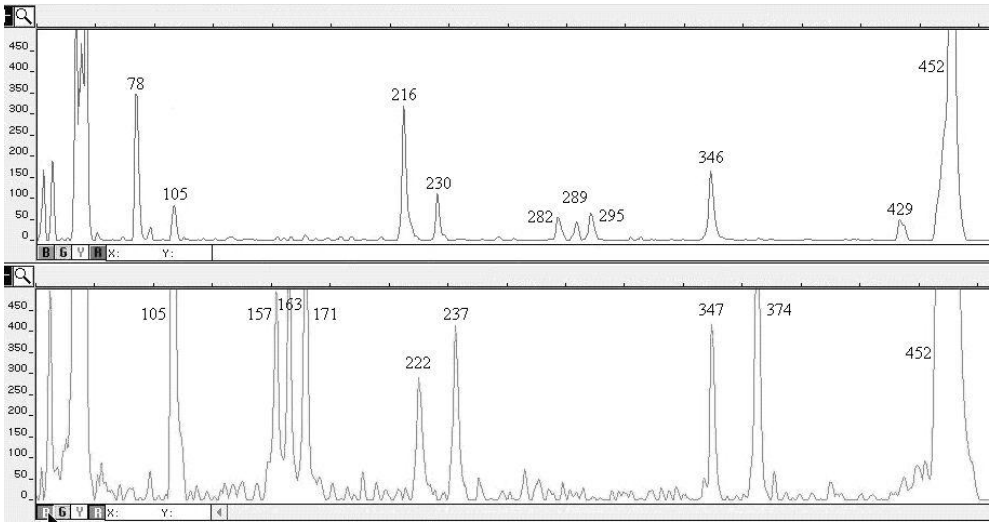
Figure 4: Polymorphisms found in the coding sequence of the *Mla* gene of four near isogenic lines *Mla1*, *Mla6*, *Mla12* and *Mla13*. The sequence shown is the coding sequence of *Mla1*, places where one or more alternative bases are found in the near isogenic lines *Mla6*, *Mla12* and *Mla13* are light grey. Mla1-3 forward and reverse primer sites are underlined.

CEL I-based mutation detection for *Mla*

In the case of *Mla*, we observed CEL I digestion products with DNA from a single variety. Normally, we would mix DNA from a standard DNA, which in this case could be *Mla1*, together with a tester DNA and use the resulting pattern to detect whether the mutant contained allelic differences. However, when the DNA from the variety possessing *Mla1* was subjected to CEL I-based mutation detection alone, a specific fragment pattern was observed. The same was true for all of the varieties, a specific fragment pattern was observed without mixing the DNA with standard DNA. This indicates that nearly identical copies of the sequence amplified by the primers are present in the respective genomes.

A summary of the observed fragment patterns is shown in Table 4. The fragment pattern detected is rather complex, with multiple mismatches for each of the lines. In the CEL I reaction the enzyme cuts only a small portion of the heteroduplices such that most of the full-length PCR product is not digested. Therefore, multiple mismatches in the same substrate can be detected. We observed 5-10 point mutations per 451 nucleotides which correspond to 98-99 % identity at the nucleotide level.

Figure 5: Mutation detection analyses of RS20-1 (*Mla18*). Vertical axis, relative fluorescence units; horizontal axis, fragment length in nucleotides. The upper panel shows the fragments of the 6-FAM-labelled strand, the lower panel shows the fragments of the VIC-labelled strand. Fragments of sizes 78, 105, 216, 230, 282, 289, 295, 346 in the upper panel correspond to the fragments 374, 347, 237, 222, 171, 163 and 157 and 105 respectively. Fragment 429 in the upper panel did not have a detectable counterpart in the lower panel, since it fell into the noisy region below 88 nucleotides. In both panels the full length PCR product of 452 nucleotides is observed.



As an example, Figure 5 shows the electropherogram of the mutation detection analysis of line RS20-1 with the powdery mildew resistance gene Mla18. With the forward primer we observed fragments 78, 105, 216, 230, 282, 289, 295 and 346 nucleotides, which correspond to the fragments observed with the reverse primer of 374, 347, 237, 222, 171, 163, 157 and

105 nucleotides, respectively. In Table 4, we have omitted fragments below approximately 90 nucleotides, since these fell into the 'noisy' region of the gel.

Table 4: EcoTILLING fragments observed for the different *Mla* variants

				Fragment sizes (labelled 6-FAM / VIC)													
				105 / 347	109 / 343	138 / 314	152 / 300	165 / 287	215 / 237	230 / 222	282 / 170	289 / 163	295 / 157	335 / 117	347 / 105	362 / 90	364 / 88
Grp	**Lines**	**Gene(s)**	**Donor**														
1	P04B	*Mla7*	Lyallpur 3645	▲			▲			▲	▲	▲			▲		▲
	P05	*Mla7*	Lyallpur 3645	▲			▲			▲	▲	▲			▲		▲
	P04A	*Mla7, Ml-k*	Lyallpur 3645	▲			▲			▲	▲	▲			▲		▲
	P07	*Mla9, Ml-k*	Lyallpur 3645	▲			▲			▲	▲	▲			▲		▲
	P09	*Mla10*	Iso 12 (Durani C.I. 6316)	▲			▲			▲	▲	▲			▲		▲
	P10	*Mla12*	Emir (C.I. 11790)	▲			▲			▲	▲	▲			▲		▲
	OPTIC	*Mla12*	Emir (C.I. 11790)	▲			▲			▲	▲	▲			▲		▲
	P11	*Mla13*	Rupee (C.I. 4355)	▲			▲			▲	▲	▲			▲		▲
2	P02	*Mla3*	Ricardo (C.I. 6306)	▲			▲			▲	▲	▲			▲		
	P13	*Mla23*	HOR1402	▲			▲			▲	▲	▲			▲		
3	D*1B-86B	*Mla19*	*H. spontaneum* 1B-86B	▲						▲	▲	▲		▲	▲		
	D*1B-151	*Mla28*	*H. spontaneum* 1B-151	▲						▲	▲	▲		▲	▲		
4	P01	*Mla1*	Algerian (C.I. 1179)		▲	▲				▲	▲	▲			▲		
	Pallas	*Mla8*	Heils Hanna (C.I. 682)		▲	▲				▲	▲	▲			▲		
	P12	*Mla22*	HOR1657		▲	▲				▲	▲	▲			▲		
5	P03	*Mla6, Mla14*	*H. spontaneum* 204							▲		▲			▲		
	RS70-29*Picc	*Mla34*	*H. spontaneum* RS70-29							▲		▲			▲		
6	P08B	*Mla9*	Monte Cristo (C.I. 1017)					▲		▲		▲			▲		
7	D*1B-20	*Mla26*	*H. spontaneum* 1B-20	▲						▲	▲				▲		
8	RS170-47*Kieb.B	*Mla17*	*H. spontaneum* RS170-47	▲					▲	▲	▲	▲			▲		
9	RS20-1*Kieb.B	*Mla18*	*H. spontaneum* RS20-1	▲					▲	▲	▲	▲	▲		▲		
10	Turkey290	*Mla31*	Turkey 290	▲					▲	▲	▲	▲		▲	▲	▲	
11	110-4*Sonja	*Mla29*	*H. spontaneum* 110-4	▲						▲	▲	▲		▲	▲		
12	D*1B-54A	*Mla16*	*H. spontaneum* 1B-54B	▲						▲	▲			▲	▲		
13	RS90-13*Kieb.B	*Mla33*	*H. spontaneum* RS90-13	▲			▲			▲	▲				▲		▲

DISCUSSION

We have successfully implemented a high-throughput method to discover and detect point mutations in barley genomic DNA, using CEL I-based heteroduplex detection on an ABI PRISM^{TM} 377 DNA Sequencer. Most of the TILLING and EcoTILLING experiments published (Colbert et al. 2001, Wienholds et al. 2003, Smits et al. 2004, Comai et al. 2004, Slade et al. 2005) used a LI-COR detection system or an HPLC system (Caldwell et al. 2004) for the final fragment detection. We have shown that the ABI fragment detection systems is equally suitable for this purpose, as shown by Perry et al. (2003). A capillary system would be even more convenient compared to the gel-based system we used, because the large amounts of primers we used interfered with neighbouring lanes which would be less problematical in a capillary system. This would not only speed up the runs but also make automatic data analysis easier.

Selection of primers should be performed in the way that the polymorphism does not fall into the 'noisy' region 0-100 nucleotides from the primer binding site. The fluorescence primers together with the degraded PCR products, where the fluorescence dyes are attached, cause the noisy signal in the lower end of the gel. For the short 451 nucleotide fragment we could detect the mutation sites with an accuracy of ±1 nucleotide. The full length PCR product was always detected as a 452 nucleotide fragment. The explanation for this is probably that Taq polymerase tends to add an A to the end of PCR fragments, which makes it one nucleotide longer than the expected fragment size (Clark 1988).

We have good experience with PCR product sizes ranging from 451 to 837 base pairs. The longer the product size, the fewer primer pairs are needed to cover a large gene such as *mlo.* In our case we used five primer pairs to cover the entire coding region. For *mlo* the procedure is an efficient tool for rapid identification of genotypes as an alternative to sequencing.

For *mlo,* we detected DNA polymorphisms between genomes when comparing WT *Mlo* to each of the *mlo* mutants. For *Mla*, we detected DNA polymorphisms within the genome for each barley variant. The additional CEL I digestion products we observed in the case of *Mla,* indicate that one or more highly similar paralogs of the *Mla* sequence might exist in the barley genome. The *Mla* region of *Hordeum vulgare* ssp. *vulgare* cultivar 'Morex' has been sequenced (Wei et al. 2002). Although 'Morex' lacks a known *Mla* resistance specificity, four resistance gene paralogs *RGH1a, RGH1bcd, RGH1e* and *RGH1f* have been found. Also the resistance genes *Mla1*, *Mla6* and *Mla13* have been shown to have paralogs and have been named *Mla1-2, Mla6-2* and *Mla13-2* (Halterman and Wise 2004). We observe 5-10 point mutations in an EcoTILLING reaction with the pure line within the 451 base pair region, which corresponds to 98-99% identity. This suggests that highly identical paralogs of the gene might be present in the barley genome, either clustered with the sequenced *Mla* gene or at a completely different location. This hypothesis is supported by findings from classical experiments with different isolates of powdery mildew. Jørgensen (1994) found presence of

additional genes for example in the case of 'Arabische'/'Emir' possessing *Mla12* + *MlEm2* genes. Perhaps this will be clarified when the barley genome is completely sequenced.

The EcoTILLING fragment patterns shown in table 4 for the *Mla* gene also pinpoint problems that can arise in TILLING/EcoTILLING. While single genes in homozygous diploid organisms like the *mlo* gene are perfectly suitable for this method, the use of TILLING in heterozygote or polyploid organisms or for genes with very similar paralogs in the same genome is more complicated. Nevertheless as shown for a polyploid organism like wheat (Slade et al. 2005) it is possible to circumvent this problem by highly specific primers. Based on our EcoTILLING results on the *Mla* gene supplemented by additional sequencing, specific primers can be designed and amplification of a single product might then be possible. In the case of a heterozygous organism, a prior EcoTILLING reaction with only the line itself will help to identify the two alleles present. The absolute limit of the technique will be reached if two of the factors mentioned above will coincide, for example highly similar paralogs in a heterozygous organism.

The fragment pattern we observed for the *Mla* gene, did not disturb our identification of the respective alleles. If the respective paralogs are clustered with the gene of interest it can be expected that the linkage between the paralogs will be very rarely broken. In the case of *Mla* the hypothesis of clustered paralogs is more likely than the hypothesis of distributed paralogs as we cannot distinguish some of the 'older' alleles (Jørgensen 1994). In the case of weak linkage we would expect new combinations of paralogs and thereby new fragment patterns.

We have used the fragment patterns to distinguish the varieties from each other. Out of the 25 natural barley variants, some had the same fragment pattern and grouped together (groups 1-5, table 4), whereas most of the newly identified alleles from *Hordeum vulgare* ssp. *spontaneum* had an individual pattern which will make identification possible.

Using EcoTILLING as a functional marker system has several advantages. Compared with SNP markers that detect specifically only one point mutation, EcoTILLING reveals multiple point mutations and small insertions and deletions. It could be described as Multiple Nucleotide Polymorphism (MNP) marker. Thereby a larger number of different alleles at a given locus can be studied at once. Additionally, it is not necessary to have complete knowledge of the entire sequence in several individuals. Finally, EcoTILLING also opens up further opportunities for designing SNP markers as it helps to identify polymorphic sites in organisms.

Compared to the classical determination of the disease resistance alleles present in a line through infection with specific isolates, EcoTILLING offers two main advantages: (a) there are a growing number of new alleles for powdery mildew resistance genes for the *Mla* locus, mainly originating from *Hordeum vulgare* ssp. *spontaneum* lines from Israel (Jahoor and Fischbeck 1993, Kintzios et al. 1995). The identification of all these new specificities is very difficult and requires the use of isolates from Israel. Since in Europe no powdery mildew population with virulence against Israeli resistance genes exists, special care has to be taken while working with those isolates to avoid an outbreak and propagation of the respective

pathotypes. Here, EcoTILLING offers a relatively easy and appropriate alternative for identification of resistance genes with help of host-pathogen interaction. (b) When several resistance genes against one disease are combined in a line to obtain a more durable resistance (pyramiding), it is very difficult to determine the single highly effective resistance genes by isolates. In the case of a combination of the *mlo*-resistance with other powdery mildew resistance, particularly originating from *Hordeum spontaneum* genes, it is impossible to use the classical method since no virulence against *mlo* exists. Here, linked markers for the respective genes have been proposed. EcoTILLING markers are even more appropriate as they have the advantage of higher specificity through a higher number of possible fragment patterns and higher reliability through the lack of linkage break.

We showed that EcoTILLING is a powerful tool for the detection of SNP in the case of *mlo* and as a useful marker system in the case of *Mla*. As a marker system it combines the advantages of a functional marker, as it is based on the gene of interest itself, with a very high number of marker alleles, as every SNP on the amplified sequence results in a distinct fragment changing the overall fragment pattern.

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