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DHPLC technology as a high-throughput detection of mutations in a durum wheat TILLING population

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Introduction

Durum wheat (*Triticum turgidum* ssp. *durum*) has a tetraploid genome (AABB genome) with genes in two homoeologous copies, a high proportion of repetitive DNA regions and a low gene density. Current tools for the discovery of sequence alterations in target genes rely on reverse genetic approaches such as the TILLING (Targeting Induced Local Lesions IN Genomes) technique. TILLING is based on the detection of chemical induced mutations in heteroduplex mainly using endonuclease (*CelI* enzyme), standard gel electrophoresis, fluorescent capillary electrophoresis and/or LI-COR techniques. Alternatively, DHPLC approach reveals the presence of genetic variation by the differential retention of the homo- and heteroduplex DNA molecules on ion-pair reversed-phase high-pressure liquid chromatography supported under partial denaturing conditions.

Objectives:

- ✓ optimization of DHPLC technique in TILLING procedure;
- ✓ mutation density evaluation by screening a subset of 1,140 mutagenized lines;
- ✓ assessment of the performance of *CelI* cleavage assay/gel method and DHPLC technique in SNP detection

Material and methods

TILLING library: 1,140 mutant lines derived from a durum wheat TILLING population (cultivar MarcoAurelio) mutagenized with EMS (0.7-0.80% EMS).

Candidate genes: lycopene epsilon-cyclase (*Lcy-ε*) (GeneBank: EU649785, EU649786) and lycopene beta-cyclase (*Lcy-β*) in A and B genomes (genes involved in carotenoid biosynthesis). All sequences were subjected to bioinformatic analysis by Primer3.0 and CODDLE.

DHPLC analysis: the DHPLC analysis was performed using the Transgenomic WAVE system under size and/or melting temperature procedures.

Optimization of TILLING procedure through DHPLC method

In order to test the best performance of DHPLC procedure scanning method for SNPs in mixed PCR amplicons, we evaluated 1 SNP in *Lcy-ε* gene (A genome) carried by two Italian durum wheat genotypes (Saadi and Casanova cultivars) in exon 5 and corresponding respectively to A/G in homozygous status. We used two genome primer combinations (**Fig. 1**): PC35-PC40 (1,193 bp) and PC133-130 (417 bp).

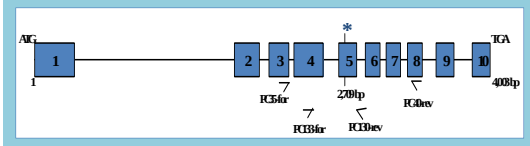


Fig. 1. Exon/intron organization of the *Lcy-ε* gene (A genome) and location of primers to screen.

CelI assay

We first adjusted the *CelI* enzyme volumes of 0.2, 0.5, 1 and 2 µl for 500 ng of PCR product from PC35-PC40 primers on agarose gel (**Fig.2**).
The lowest enzyme assay (0.2 µl) could be used as a high-throughput mutation detection method on agarose gel while the latter conditions were used to test *CelI* performance on DHPLC instrument.

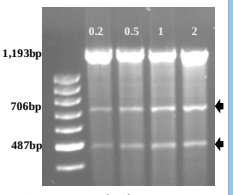
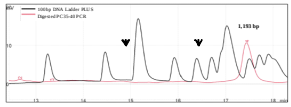


Fig. 2. *CelI* cleavage assay.

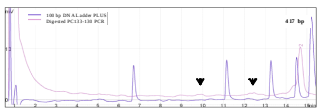
CelI/DHPLC

PC35-PC40 and PC133-130 amplicons from cv Saadi and Casanova DNAs in homo- and heteroduplex status were heated/cooled down, digested with *CelI* enzyme and loaded on DHPLC in UV and Fluorescence analysis.

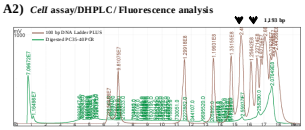
A1) *CelI* assay/DHPLC/UV analysis



B1) *CelI* assay/DHPLC/UV analysis



A2) *CelI* assay/DHPLC/Fluorescence analysis



B2) *CelI* assay/DHPLC/Fluorescence analysis

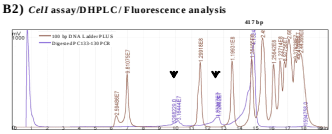


Figure 3. Comparisons of *CelI*/DHPLC analysis using UV detector (A1-B1) and fluorescent detector (A2-B2).

DHPLC method in melting temperature

The criteria of DHPLC in melting temperature analysis consisted in high PCR product yields (~500 ng), fragment size ≥ 200 and ≤ 800 bp and a minimum distance of 50 bp between primers and SNP location. According to this, we focalized on PC133-130 PCR products.

Melting curves were simulated using the Transgenomic software to determine whether any significant shifts in T_m could be predicted for the amplicon (**Fig. 4**).

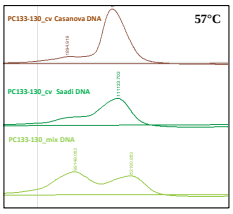


Figure 4. Outline of SNP detection by heteroduplex analysis of PC133-130 amplicons.

Even though the heteroduplex molecules were still visible up to the pool ratio of 1:6, the 4-fold pooling size was chosen for the M_2 DNA arrangement.

The DHPLC protocol optimization also involved the adjustment of sample DNA concentration in terms of pool size. Exactly PC133-130 PCR homoduplex products were mixed together in proportions of 2, 4, 6 and 8 fold pools and loaded on DHPLC (**Fig. 5**).

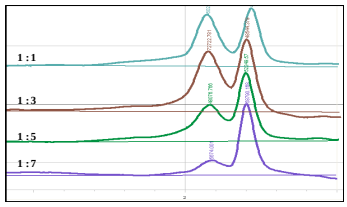


Figure 5. Evaluation of pool capacity for SNP detection.

Mutation density evaluation and comparison between CelI/gel procedure and DHPLC technique

According to Uauy et al. (2009), the applied TILLING strategy was based on: an initial screen of four-fold DNA pools to identify the mutant one and a second screen of two-fold DNA pools to detect the mutant individual for sequencing. The molecular analysis of 1,140 mutant wheat lines has been focused on two low-cost and fast methods: the CelI cleavage assay/agarose gel detection and the DHPLC technique reviewed in melting temperature condition (**Tab.1**). 26 heterozygous loci were identified by both procedures. All mutant selected genotypes were confirmed by direct sequencing.

Tab.1. Mutation frequencies obtained through the DHPLC analysis.

					Mutation Frequency	
Gene	Genome	Amplicon size	Number of screened plants	Mutations	DHPLC	*CelI*/agarose detection
Lcy-β	A	464	1,140	17	1/31	1/176
Lcy-β	B	761	1,140	8	1/108	1/167
Lcy-ε	A	1,527	1,140	17	1/102	1/138
Lcy-ε	B	1,220	1,140	21	1/84	1/148
Total/mean		**3,972**	**4,560**	**63**	**1/71**	**1/168**

The average mutation frequencies for all genes appeared different suggesting the dissimilar sensitivity of the two screening techniques. In addition, within of DHPLC screening, the mutation frequency rate changed with slight differences depending on the differences of GC content, sequence and length of the target regions.

Conclusions

✓TILLING is a powerful method to induce and identify novel genetic variation in wheat genes. The polyploid nature of wheat results in good tolerance to high mutation densities. The estimated mutation density in the tetraploid TILLING population was approximately 1 mutation per 71 kb.

✓The DHPLC technique provides to be an useful tool in SNP detection when based on melting temperature changes of heteroduplex molecules. Less reproducibility has been showed combing *CelI* assay and sizing program (in UV and fluorescent condition).

✓CelI/agarose gel method and DHPLC have been proven to improve the throughput of SNP detection in plants at reasonable cost. The choice will be ultimately determined by the requirements, potentialities and drawbacks of each technique based on the amplicon length, the knowledge of the complete sequence and the costs of each method.

✓This study is a beautiful example of DHPLC technology application and shows an alternative tool to current strategies of SNP detection based on genotyping array.