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Blind mapping of genic DNA sequence polymorphisms in *Lolium perenne* L. based on high resolution melting curve analysis

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1. Introduction and objectives

Genetic linkage mapping is a key tool for the development of superior varieties via marker-assisted selection and for gene isolation via map-based cloning. The focus of DNA markers used for mapping has recently changed towards gene derived simple sequence repeat (SSR) and single nucleotide polymorphism (SNP).

Genotyping of length polymorphisms in SSRs by gel- or capillary electrophoresis is inefficient or requires labelled primers and additional steps following PCR. For SNP genotyping, single nucleotide primer extension (SNuPe), EcoTILLING or multi-parallel technologies based on mass spectroscopy, pyrosequencing or the Illumina GoldenGate platform are among the most widely used techniques. However, all these post PCR technologies are expensive, time consuming to implement and run, or require detailed knowledge on allelic sequence information. Therefore, a simple, fast and cheap method to genotype not only a particular marker type but any DNA sequence polymorphisms (or combinations of polymorphisms) is needed.

High resolution melting curve analysis (HRM) measures dissociation of double stranded DNA from a PCR product amplified in the presence of a saturating fluorescence dye. In this study, HRM was evaluated for the ability to discriminate not only specifically addressed SSR and SNP polymorphisms, but to differentiate alleles in a PCR fragment, in which type and number of allelic DNA polymorphisms are unknown. Moreover, a novel HRM-based approach to map any DNA sequence polymorphism in plants without allelic sequence information – referred to as blind mapping - is introduced and used for mapping *LpVRN3*, a candidate gene for vernalization requirement in perennial ryegrass.

2. Materials and methods

2.1 Genic SSRs, SNPs and Indels

Genic SNPs, InDels and SSRs of recently published perennial ryegrass ESTs (Studer et al. 2008) were selected for short amplicon genotyping by HRM. The loci represent different types and combinations of sequence variation as well as different complexity levels of segregation patterns with up to four segregating alleles in the VrnA mapping population (Table 1).

Table 1: Description of amplified polymorphisms and segregation patterns in VrnA of loci used for high resolution melting curve analysis (HRM).

Marker/locus name	Polymorphism	Segregation pattern		Gene description [b]
SNP 927_2	SNP	hkxhk	1:2:1	BiRP1 ribosomal protein [*Bromus inermis*]
G05_071	SSR	abxcd	1:1:1:1	Aux/iaa gene family [*Oryza sativa*]
G05_088	SSR, InDel, 4 SNPs	egxef	1:1:1:1	Triosephosphate translocator [*Oryza sativa*]
LpVRN3	2 SNPs [a]	nnxnp	1:1	*LpVRN3* [*Lolium perenne*]

[a] Allelic sequences were not available at the time of HRM
[b] Gene description is based on the best BLASTX hit against the nr protein database of GenBank using the BLASTX programme

2.2 Plant material

The VrnA F2 perennial ryegrass mapping population along with the parents (NV#20F1-30 and NV#20F1-39) and the grandparents (Falster and Veyo) were used for blind mapping. Genomic DNA was extracted from fresh leaf tissue, quantified using a Synergy™2 Multi-Detection Microplate Reader (BioTek® Instruments, Inc., VT, USA) and diluted to a concentration of 10 ng/µl.

2.3 Primer design

Primers for SNP genotyping (SNP 927_2) were designed to directly flank the SNP, resulting in a product length of 43 bp. For G05_071 and G05_088, primers amplifying fragment lengths between 83 and 200 bp were designed according to the following criteria:

- 18 bp < length of primers < 24 bp
- 57°C < annealing T < 61°C, delta T <1 °C
- 40 % < GC content < 70 %

2.4 PCR conditions

PCR was performed in a total volume of 10 µl using 1X LightScanner high sensitivity master mix (Idaho Technology, Inc, UT, USA), containing LCGreen® PLUS and 0.05 mM of each internal oligonucleotide calibrator. In addition, each reaction contained 0.10 mM of each forward and reverse primer, and 20 ng of DNA. In order to avoid evaporation of the PCR mix during PCR amplification and HRM, reactions were covered with 15 µl mineral oil. All reactions were performed in duplicate.

The PCR amplification was conducted in a PTC-225 Peltier Thermal Cycler (MJ Research Inc., Watertown, MA, USA) as follows: initial denaturation of 2 min at 95°C, followed by 40 cycles of 30 s at 94°C, 30 s at the optimal annealing temperature for each primer pair and a 30 s elongation step at 72°C. For heteroduplex formation, a final cycle of 30 s at 94°C and 30 s at 25°C was added.

2.5 HRM analysis

HRM was performed using the LightScanner Instrument (96-well plate format) and the LightScanner® and Call-IT® software modules (Idaho Technology, Inc). HRM analysis was carried out following PCR at temperatures from 70°C to 90°C at steps of 0.05°C, each step with a 1s hold.

3. Results

At each locus, HRM revealed two, three or four different curve shapes in VrnA depending on the complexity levels of the segregation patterns (Figure 1, A-D). The congruence between melting profiles of replicated samples from identical genotypes was high. The *LpVRN3* gene mapped to position 33 cM on LG 7 in VrnA.

Figure 1, A Normalized melting curves and segregation pattern of SNP 927_2

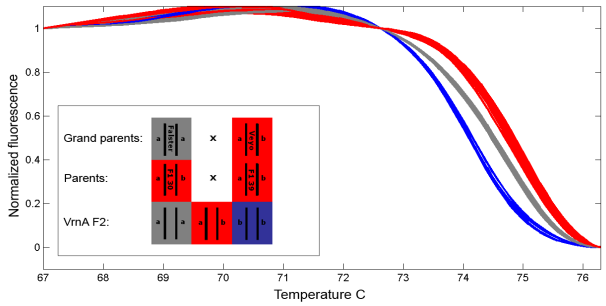

Figure 1, B Normalized melting curves and segregation pattern of G05_071

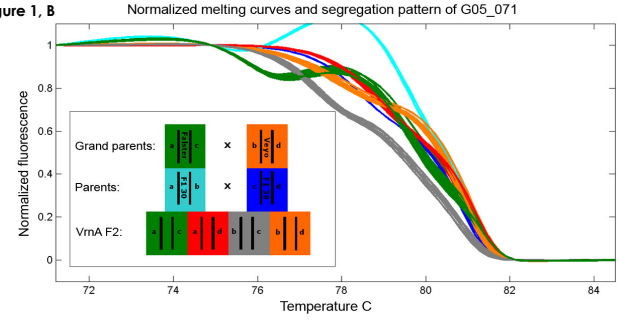

Figure 1, C Normalized melting curves and segregation pattern of G05_088

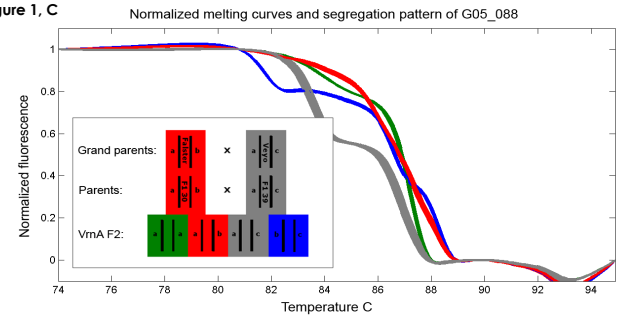

Figure 1, D Normalized melting curves and segregation pattern of *LpVRN3*

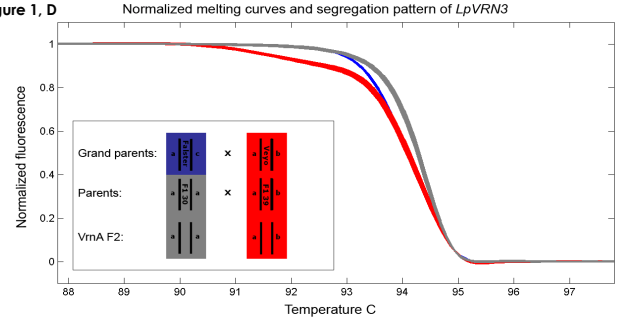

Figure 1, A-D: The normalized melting curves and the corresponding segregation pattern of genic loci in VrnA. Groups exhibiting similar melting profiles are represented with similar colours.

4. Summary and conclusions

- HRM is an extremely powerful technique for genotyping any type of sequence polymorphisms in plants
- Blind mapping by HRM does not require allelic sequence information and proved successful to map any type of sequence polymorphisms in the perennial ryegrass F2 mapping population VrnA
- Using blind mapping, *LpVRN3*, a candidate gene for vernalization response in perennial ryegrass, was mapped on linkage group 7 in VrnA

5. Reference

Studer B, Fiil A, Jensen LB, Asp T (2009) "Blind" mapping of genic DNA sequence polymorphisms in *Lolium perenne* L. by high resolution melting curve analysis. Mol Breed: in press