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Discovery of *Dhn3* variants in wild barley (*Hordeum spontaneum*) by high-resolution melting (HRM) technology

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Abstract: The analysis of allelic variation in model plant species and their wild relatives, such as *Hordeum vulgare* and *Hordeum spontaneum*, is useful for relating genetic determinants and important phenotypic traits such as stress tolerance. High resolution melting (HRM) analysis is a cost-effective, rapid, and high-throughput assay for mutation screening and genotyping without sequencing. The present study describes an HRM analysis of natural sequence variation within *Dhn3* alleles from different *H. spontaneum* accessions. Small PCR-derived amplicon assays were developed for exon 1 and exon 2 of *Dhn3*. The efficiency of the HRM procedure was affected by various factors, including specificity and efficiency of PCR, amplicon length and position, and DNA template quality. In addition to these factors, the use of PCR product rather than genomic DNA in HRM increased the quality of melting curves, thus affecting the accuracy and sensitivity of the assay. HRM classified 5–6 groups of variants carrying deletion mutants and single and multiple SNPs consistent with the sequencing data. 18-bp deletion variants were distinguished from the reference sample according to HRM analysis of 207-bp fragment of exon 1. A/T conversions were difficult to discriminate variants, whereas A/G or T/C transitions were easily grouped because they required high T_m differences. The conditions and parameters for an optimized HRM assay were outlined, and its potential application in allelic variation research of stress-related genes was discussed.

Key words: High-resolution melting, wild barley, *Dhn3*, *Hordeum spontaneum*

1. Introduction

Natural polymorphism discovery in genes of interest represents a powerful means of elucidating gene function, breeding, and conservation of natural diversity. The analysis of allelic variation in wild (*Hordeum spontaneum* C.Koch) and cultivated (*Hordeum vulgare* L.) barley is particularly useful for relating genetic determinants and important phenotypic traits such as stress tolerance. High-resolution melting (HRM) analysis has become a preferred method for fast screening allele mutations and diagnostic purposes in the field of human genetics, plant science, and pathology (Hofinger et al., 2009; Cousins et al., 2012; Er and Chang, 2012). This technique was first developed for screening mutations in human genes by obtaining high-resolution amplicon melting curves (Wittwer and Reed, 2003). The characterization of DNA samples is based on monitoring changes in fluorescence after dissociation of PCR amplicons in the presence of a double-strand DNA binding dye (Hofinger et al., 2009). Once optimized, SNP level variations in amplicons are revealed through differences in melting transitions in a close-tube system with high accuracy (Montgomery et al., 2007). This method is considered fast, sensitive, and low-cost in comparison to traditional assays such as single strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), and denaturing high-performance liquid chromatography (DHPLC) (Hofinger et al., 2009). Even though HRM analysis has been widely used in clinical applications, it was recently adapted for genotyping in cereal species, including barley (Lehmensiek et al., 2008; Hofinger et al., 2009), wheat (Botticella et al., 2011; Sestili et al., 2015), and rice (Li et al., 2011). Allelic variants of *eukaryotic translation initiation 4E* (*HvelF4E*) gene have been investigated in barley with HRM analysis for the potential application in targeting induced local lesions in genomes (TILLING) (McCallum et al., 2000; Hofinger et al., 2009). HRM technology has been recently used to discover SNP and indel variations of stress-related genes (Mondini et al., 2011).

Dehydrins are classified as the group 2 late embryogenesis abundant (LEA) proteins that are upregulated during embryo maturation, stress exposure,

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and exogenous applications of abscisic acid (ABA) (Choi et al., 1999; Tommasini et al., 2008). Dehydrins were found to be associated with salinity (Ruibal et al., 2012; Sadder and Al-Doss, 2014), cold (Yang et al., 2012), and drought (Tripepi et al., 2011; Karami et al., 2013) tolerance in different plant species. Thirteen dehydrin genes have been identified in barley and genetically analyzed in cultivar Dicktoo at both DNA and protein levels (Choi et al., 1999; Rodriguez et al., 2005). *Dehydrin3 (Dhn3)* is one of the key members of this family, showing significant expression under drought, low temperature, ABA treatments, and photoperiodic changes (Park et al., 2006; Guo et al., 2009; Karami et al., 2013). *Dehydrin* family genes in *H. vulgare* were proposed to be generated after several transposition and duplication events (Choi et al., 1999). However, polymorphisms in *Dhn3* locus are not sufficiently known in barley and its wild progenitor, *H. spontaneum* C.Koch, which is widely distributed in Turkey and other parts of the Fertile Crescent. In this research, our group sequenced dehydrin-encoding gene *Dhn3* from *H. spontaneum* populations to investigate nucleotide diversity (unpublished results). In the present study, HRM analysis was tested and optimized for *Dhn3* exonic sequences to investigate its efficiency for further investigations of *H. spontaneum* populations.

2. Materials and methods

2.1. Plant materials

Sixteen *H. spontaneum* accessions, originating from western and southeastern parts of Turkey, and *Hordeum vulgare* L. cv. Tokak157/37 (Table 1) were used in this study. Genomic DNA was isolated from 100 mg of homogenized leaves from single plants by using the CTAB method (Weining and Langridge, 1991).

2.2. Amplicon preparation

Dhn3 gene in barley consists of two exons and one intron (Figure 1). Four genomic DNA-derived regions were selected for HRM using the *Dhn3* gene isolated from Tokak157/37 as a reference. Primer sequences and corresponding positions on amplicons are given in Table 2.

Table 1. Summary of nucleotide changes in *Dhn3* alleles based on the first and second exon sequences by Sanger sequencing, and comparison to HRM grouping in 16 *H. spontaneum* accessions and Tokak157/37 cultivar as reference**.** AARI: Aegean Agricultural Research Institute, DPPRI: Diyarbakır Plant Protection Research Institute. [a]HRM grouping was achieved by a sensitivity value of 0.5 according to Gene Scanning Program 1.5.

Accessions	Origin	Provider	Position/SNPs	Groups by HRM [a]	Position/SNPs	Groups by HRM [a]
			Exon1b (207 bp)		*Exon2b* (176 bp)	
Tokak157/37	-	İstanbul Univ.	-	1	-	1
TR4982	Çanakkale	AARI	141–158 (18-bp deletion), 240 A>T	3	504 G>C, 609 A>G, 621 T>A	3
TR31623	Mardin	AARI	240 A>T	1	-	1
TR40812	Gaziantep	AARI	240 A>T	1	-	1
TR47002	İzmir	AARI	141–158 (18-bp deletion), 240 A>T, 270 A>G	6	478 G>A, 504 G>C	5
TR49085	Adıyaman	AARI	141–158 (18-bp deletion), 240 A>T, 285 C>T, 291T>C	2	-	1
TR50358	Diyarbakır	AARI	240 A>T	1	-	1
AA1	Diyarbakır	Dicle Univ.	240 A>T, 227 G>A, 290 C>G	5	560 A>G	2
AA2	Diyarbakır	Dicle Univ.	240 A>T	1	-	1
AA3	Diyarbakır	Dicle Univ.	240 A>T	1	474 A>G	2
K169	Diyarbakır	DPPRI	240 A>T	1	507 C>T	4
K394	Diyarbakır	DPPRI	240 A>T	1	-	1
K1239	Diyarbakır	DPPRI	240 A>T, 180 C>T	1	-	1
LH1	Batman	DPPRI	101 A>T, 240 A>T	1	553 G>A	1
LH2	Batman	DPPRI	116 A>G, 145 A>G, 290 C>G, 240 A>T,	4	-	1
LH3	Batman	İstanbul Univ.	240 A>T	1	-	1
LK9	Diyarbakır	İstanbul Univ.	240 A>T	1	-	1

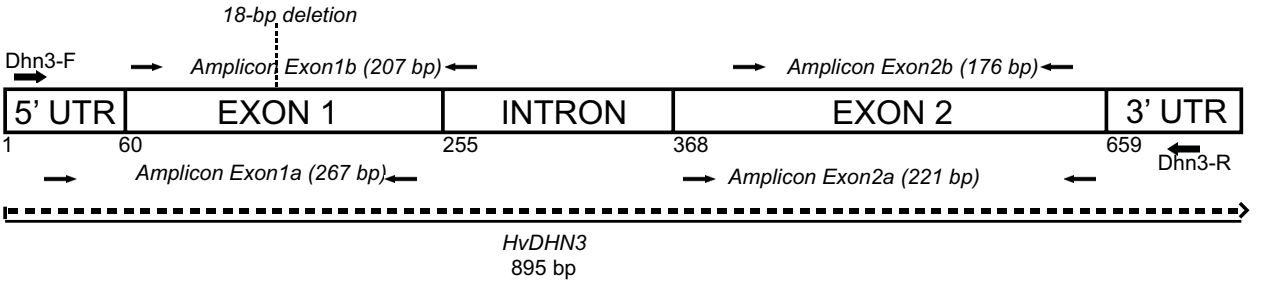

Figure 1. Schematic diagram of barley *Dhn3* gene according to Tokak157/37 and NCBI accessions of cv. Dicktoo (AF043089.1) (Choi et al., 1999). The arrows represent the positions of the primers used in the study.

Table 2. Primer sequences of *Dhn3* gene for HRM analysis in wild barley.

Fragment	Primers (5'*3')	T_A (C)	Amplicon size (bp)	Position of amplicon (start/end)
Dhn3	AGGCAACCAAGATCAACACC TTCTGCAAGGTAGCCAGACC	61	692	24–715
Exon1a	GGCAACCAAGATCAACACCA AGTGCTGGCAAGAAGTAAGT	60	267	25–291
Exon1b	GCGTCGACGAGTACGGTAAC AGTGCTGGCAAGAAGTAAGT	60	207	85–291
Exon2a	GAGGAAGAAGGGCCTCAAG CCTTCTTCTCGCCAGTCC	60	221	394–614
Exon2b	GTGATCAGCAGCAGACCGG CATGATGCCCTTCTTCTCGC	60	176	447–622

2.3. PCR and HRM conditions

50–100 ng genomic DNA or PCR product of *Dhn3* were used for HRM analysis. When using PCR product as a template for HRM analysis, 50 ng of genomic DNA was used to amplify *Dhn3* allele. PCR reactions included 0.4 µM forward and reverse primers, 0.25 mM dNTPs, 2 mM $MgCl_2$, and 1 U GoTaq DNA polymerase (Promega, USA). PCR cycling conditions were programmed as initial denaturation at 95 °C for 5 min, followed by 35 amplification cycles of denaturation at 95 °C for 30 s, annealing at 61 °C for 1 min, and elongation at 72 °C for 40 s, with a final extension at 72 °C for 15 min in a thermal cycler (Bio-Rad, T100, USA). The resulting PCR product was analyzed in 1% agarose gels. HRM reactions were carried out using 96 well-plates (Roche Applied Sciences, USA). Two dilution series of first-round PCR products (250- and 500-fold) were used to optimize HRM analysis. Reactions were performed in a final volume of 10 µL containing 1 × AccuMelt HRM SuperMix (Quanta, USA), 0.25 µM of each internal primer, and diluted first-round PCR products. Amplification and melting conditions were as follows: 94 °C for 10 min, 35 cycles at 94 °C for 10 s, 60 °C for 30 s, 72 °C for 20 s, and melting at a gradient from 60 °C to 95 °C at 0.02 °C per second; 25 fluorescence acquisitions per 1 °C. Three technical replicates were used in all reactions, which were performed on a LightCycler 480 II (Roche Applied Sciences). Data were analyzed with Gene Scanning Program 1.5.

3. Results and discussion

A 692-bp fragment of the *Dhn3* gene (including exon1 and exon2) was obtained in all barley accessions from genomic DNA by PCR (Figure 2). Homogeneity in the amount of PCR products observed by agarose gel was a critical parameter for obtaining amplification curves in subsequent HRM analyses. It was not possible to use the amplicons derived from genomic DNA for HRM analysis, because the PCR products were not sufficient. This may have been due to the presence of chemical ingredients introduced by the DNA extraction procedure. Decreased sensitivity by gDNA was also observed in clinical applications of HRM (Carillo et al., 2011). Instead, a nested PCR strategy, using the amplicons of the first PCR as template, was efficiently used for HRM analysis in our study. 500-fold dilution of

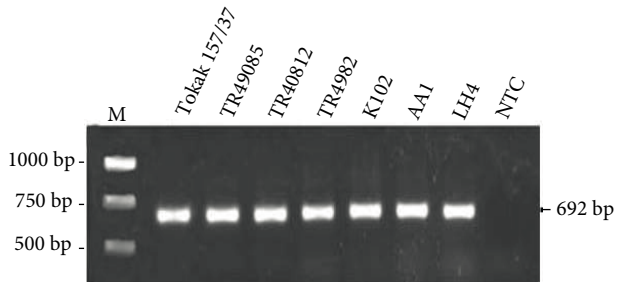

Figure 2. Amplification of *Dhn3* allele in Tokak157/37 and six *H. spontaneum* accessions, which originated from different geographical locations given in Table 2. M: (1 kb DNA ladder, Fermentas), NTC: no template control.

PCR products yielded better melting curves than 250-fold diluted samples.

Two amplicons, *exon1b* (207 bp) and *exon2b* (176 bp), were selected for polymorphism analysis, as they generated optimal normalized shifted melting curves (Figure 3). Although there was amplification with low C_t values, melting curves were not obtained by *exon1a* and *exon2a* amplicons. This result may be associated with large amplicon size (e.g., >250 bp), which has been reported as an important parameter for HRM sensitivity (Gundry et al., 2008; Hofinger et al., 2009). In contrast, the applicability of *exon1b* and *exon2b* amplicons in identifying variations was determined by normalized and shifted melting curves and temperature-shifted difference plots (Figures 3a and 3b). Aligned melting curves and difference plots of 16 accessions demonstrated the allelic variation by both amplicons (Figure 3).

To evaluate HRM predictions, both exons were verified by sequencing (Table 1). The grouping of variants in all samples was consistent with the sequencing data. A deletion of 18-bp in position 82 on the first exon was detected in three *H. spontaneum* accessions (TR47002, TR4982, and TR49085). The presence of this deletion in the first exon of *Dhn3* was previously reported in Dicktoo clone Dc15-130 (Choi et al., 1999). In total, 6 and 5 variant groups were detected in the analysis of *exon1b* and *exon2b*, respectively (Table 1). According to *exon1b* results, Tokak157/37 and 11 *H. spontaneum* accessions were clustered in Group 1, since they had similar melting curve patterns, and the rest of the *H. spontaneum* accessions were distributed between Groups 2–6. By using *exon1b* amplicon, accessions with indel and additional SNPs were clearly distinguished from Tokak157/37 (Table 1). Nine accessions carried only a conserved single SNP (A > T) at position 240, and HRM classified these accessions in the same group of the reference cultivar, which lacks this SNP (Table 1). A/T conversions have been reported as the most difficult SNP for genotyping, because they require very small T_m differences (<0.2 °C) (Venter et al., 2001) (Figure 3c). Variants carrying 2 SNPs and multiple SNPs with deletion mutations were clearly distinguished by melting curve analysis for both *exon1b* and *exon2b* (Figures 3d and 3e). The assay (*exon1b*) was able to distinguish TR47002 accession from TR4982 by a specific change in A>G, even though both genotypes carry 18-bp deletion and a conserved A >T substitution (Table 1). This was an expected result, because A>G transitions can be determined by relatively large T_m differences (>0.5 °C). We detected that 3 accessions (TR49085, AA1, and LH2) included one or two SNPs in the primer sequences. However, the presence of SNPs in primer sequences did not affect accuracy of grouping, unlike a previous report (Hofinger et al., 2009). By *exon2b* analysis, amplicons containing one (A>G, C>T) or multiple SNPs were distinguished from the reference cultivar by automatic grouping, while G>A transition in LH1 could not be differentiated from the reference sample. In *exon2b*, several variants carrying one or multiple SNPs (e.g., TR4982, TR47002, and K169) were included in different groups, indicating good sensitivity of the assay. The assay of *exon2b* grouped accessions AA1 and AA3 in the same pattern of SNP variants; however, it carried a single A>G transition in different positions. In contrast, the accession LH1 could not be distinguished from the reference sample despite the presence of the SNP G>A at position 553 in *exon2b*. In conclusion, the sensitivity of the assay was affected by the length and intragenic position of the amplicons. In this study, the evaluation of both exons was sounder than individual considerations because there were several examples, such as AA2, AA3, and K169, which were similar regarding *exon1b*, whereas those were in three separate groups of *exon2b*. Homozygosity of the locus was usually considered as a limiting factor for HRM analysis. Use of smaller amplicons has been suggested in order to detect homozygotes that display similar melting curves and T_m (Gundry et al., 2008). Therefore, homozygous mutants in clinical applications could be distinguished from wild type alleles by using very short amplicons (e.g., 42–86 bp) in length (Gundry et al., 2008). In our study, *exon2b* amplicons were carried as homozygous alleles in all genotypes, whereas TR31623, TR40812, TR47002, TR49085, AA1, AA2, AA3, K1239, and Tokak157/37 were heterozygous for 207-bp *exon1b* amplicon. This was determined by the formation of two melting peaks, as shown in Figure 3f for Tokak157/37 and AA2 accessions. In terms of heterozygosity, the genotype of exon sequences in accessions examined in this work did not affect HRM predictions.

This is the first report on the use of HRM analysis for *H. spontaneum*. Our technique provided data for distinguishing variant *H. spontaneum* accessions from *Dhn3* alleles at exon level. The method allowed a rapid

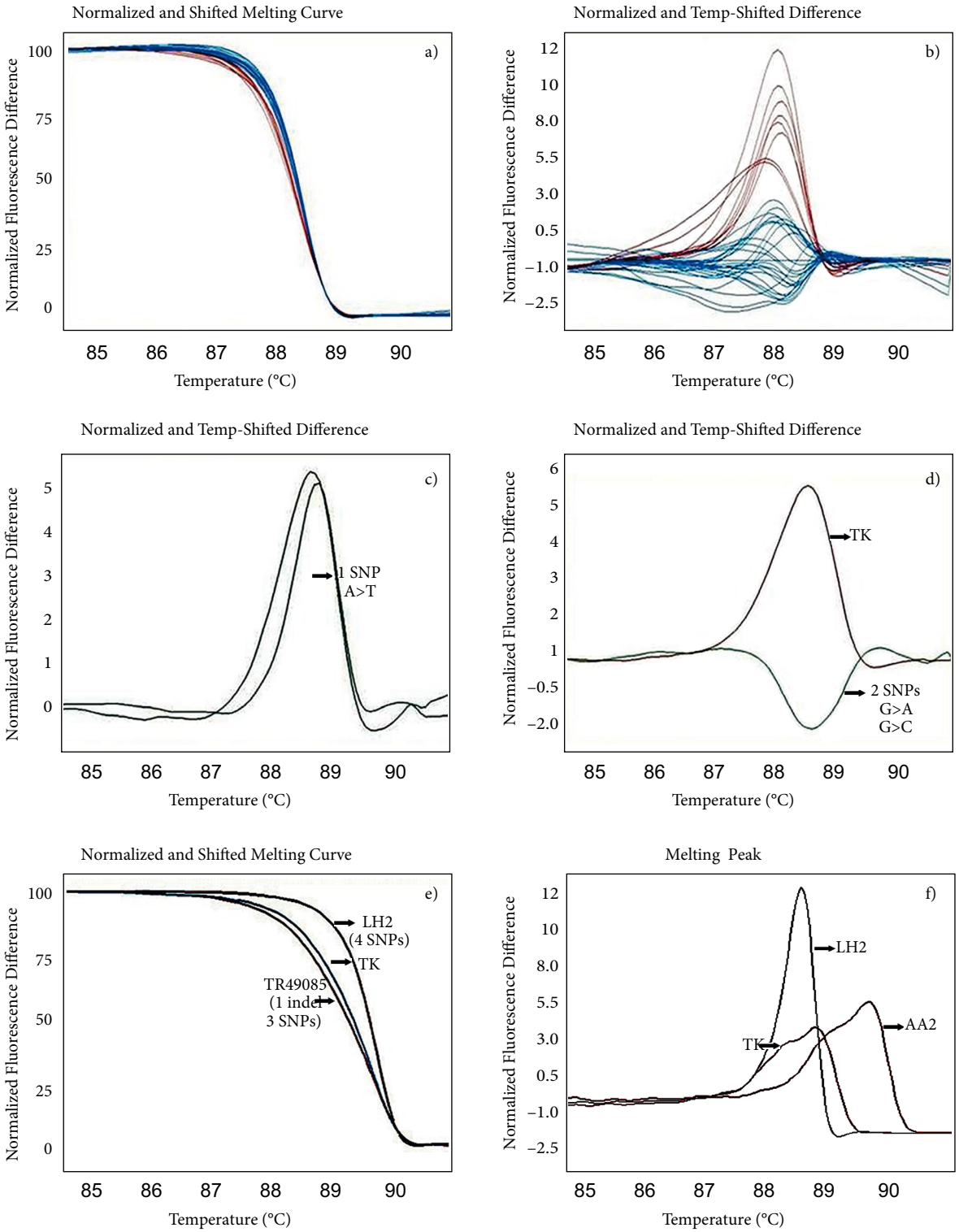

Figure 3. Optimization of HRM analysis for *Dhn3* of *H. spontaneum*. a) normalized and shifted melting; b) normalized and temperature-shifted difference plot of 16 *H. spontaneum* accessions for 176-bp *exon2b* amplicon; c) temperature-shifted difference plot of A>T substitution in variant AA3 by *exon1b* analysis; d) temperature-shifted difference plot of Tokak157/37 and TR47002 carrying double SNP substitutions of G>A and G > C; e) discrimination of variants with an indel + 3SNPs (TR49085), 4 SNPs (LH2), and Tokak157/37 by normalized and shifted melting curves; f) genotyping of *exon2b* region of *Dhn3* allele in two *H. spontaneum* accessions and TK (Tokak157/37).

screening and grouping of natural germplasm based on definite genes without sequencing data. This is advantageous when using wild accessions from large numbers of populations collected from different climates, soil types, or geographic locations. *H. spontaneum* is widely distributed in the Mediterranean, the Irano-Turanian zone, and the Fertile Crescent, thereby showing high adaptation to different habitats (Harlan and Zohary, 1966). The identification of genomic variations associated with climatic adaptations found in wild relatives has been reported to be crucial in modern crop breeding (Garg et al., 2014). Therefore, the HRM method optimized by the present study could be used for large-scale genotyping of wild barley populations to study stress biology or geographic origin of domestication.

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