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High Resolution Melting and Insertion Site-Based Polymorphism Markers for Wheat Variability Analysis and Candidate Genes Selection at Drought and Heat MQTL Loci

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Abstract: The practical use of molecular markers is facilitated by cost-effective detection techniques. In this work, wheat insertion site-based polymorphisms (ISBP) markers were set up for genotyping using high-resolution melting analysis (HRM). Polymorphic HRM-ISBP assays were developed for wheat chromosomes 4A and 3B and used for wheat variability assessment. The marker sequences were mapped against the wheat genome reference sequence, targeting interesting genes. Those genes were located within or in proximity to previously described quantitative trait loci (QTL) or meta-quantitative trait loci (MQTL) for drought and heat stress tolerance, and also yield and yield related traits. Eighteen of the markers used tagged drought related genes and, interestingly, eight of the genes were differentially expressed under different abiotic stress conditions. These results confirmed HRM as a cost-effective and efficient tool for wheat breeding programs.

Keywords: high resolution melting; ISBP markers; drought; candidate genes; QTL; MQTL; wheat variability

1. Introduction

Wheat is among the most important and widely grown crops worldwide [1] and one of the most important grain food crops in the human diet (https://www.fao.org). Wheat development and yield can be affected by abiotic stresses as drought [2–5] and heat [6,7], whose frequencies would be increased by the strong effects of the predicted climate change and global warming [8–10]. In fact, drought is considered one of the most limiting environmental factors [3,11,12], strongly affecting the growth [13,14] and production of crops, with significant reductions in the final yield of cereals, including wheat [13]. Heat stress usually affects crops during the post-anthesis period, with negative effects on final production [15] and end-use quality products [16]. These abiotic stresses are important

challenges for plant research and breeders, and plant breeding efforts have been focused on the improvement of final crops production under these limiting conditions [17].

Drought tolerance is considered a complex [18,19] and quantitative trait [14], which interacts with the environment, and possesses an additive polygenic nature [3]. Even though most of these genes have minor contributions, they are of great importance in the genetic improvement for drought tolerance [5]. Drought can also affect gene expression [20,21], both under controlled [22–25] and field conditions [26,27]. Heat stress tolerance includes plant mechanisms which occur at several levels, where the plant acquires thermotolerance to cope with high temperatures [28]. Plants heat shock response results from the reprogramming of gene expression [29] and is of great interest for studies focused on plants stress tolerance and gene expression regulation [28]. Wheat breeding programmes are necessary to ensure an improved selection of favorable alleles focused on interesting agronomic traits, as yield and quality, and also biotic and abiotic stresses tolerance [30,31].

Molecular markers can be developed and successfully applied to identify important genomic regions and major genes [32] closely related to target traits as drought tolerance [3]. In addition, public resources as Wheat Expression (http://www.wheat-expression.com) facilitate the identification of interesting candidate genes, as well as their validation [22,27]. Recent advances in genomics, and the available fully annotated wheat reference genome (IWGSC RefSeq v1) [33], allow the accurate identification of marker positions and their chromosome locations [5]. The available gene models have been used, through appropriate bioinformatic pipelines, for the identification of differentially expressed genes during drought and heat stress treatments (i.e., [22,27]).

The insertion site-based polymorphism markers (ISBP) are PCR markers designed based on the knowledge of the sequence flanking transposable element (TE) sequences, to design one primer in the transposable element and the other in the flanking DNA sequence [34]. TEs are very abundant and nested in the wheat genome, with unique (genome-specific) insertion sites that are highly polymorphic [30]. ISBP were developed for wheat genomic and genetic studies [34,35], and later used in marker-assisted selection (MAS), and as a selecting tool for new varieties in plant breeding programs [30]. The ISBP technique is a rapid and efficient way to develop single copy chromosome-specific markers from incomplete genomic sequences [30,34]. ISBP markers represent a valuable source of polymorphism, which is mostly genome specific in wheat [35], and therefore very convenient for wheat mapping applications [36]. These markers have been used to improve the wheat genome saturation [30,33,37], for genotyping and single-nucleotide polymorphism (SNP) detection [35,38], genetic diversity assessment [39], micro RNA (miRNA) coding sequences identification [40], or sequence composition analysis [41]. They were also successfully applied to develop physical or genetic maps, and to locate important agronomic traits [42,43] or resistance genes [36,44].

There are different techniques to detect the ISBP markers, as fluorescent polymerase chain reaction (PCR) and capillary electrophoresis, allele-specific PCR or melting curve analysis [35]. High resolution melting analysis has been described as a versatile and powerful analytical tool in molecular biology, characterized by its easy use, simplicity, flexibility, low cost, sensitivity, and specificity [45–47]. Briefly, this technique is based on the analysis of the PCR product's melting, by analyzing the fluorescence (due to an intercalated dye) broadcast level as response of a specific increasing temperature ramp [48]. ISBP was firstly carried out by Paux et al. [30] using wheat chromosome 3B markers and melting curve analysis as an alternative to agarose gel electrophoresis. ISBP marker detection using HRM analysis was later performed for resistance loci assessment in bread wheat [49]. The HRM technique has also been used to detect other molecular markers (i.e., SNP, expressed-sequence tag (EST), simple-sequence repeat (SSR), or insertion-deletion (InDeL)). HRM applications in wheat and closely related species as barley and *Aegilops*, include the characterization of InDeL and SNP markers involved in drought and salt tolerance [50]; the detection SNP markers [51,52] and mutations [53–56]; and the mapping of markers linked to resistance genes [57–59].

ISBP markers have been developed for all wheat chromosomes [33,35,40,41,43]. Chromosome 3B [30] contains loci for grain yield, kernel length, plant height, and related traits [60]. Chromosome

4A [33,61] harbors several QTLs related to biotic and abiotic stresses tolerance, agronomic traits as grain yield and quality, and regulation of physiological traits as plant height, maturity, or dormancy [62–70]. This chromosome represents an important target in plant breeding, marker design for variability analysis and candidate genes assessment. In this study, ISBP markers from wheat chromosome 3B [30] and 4A were used to develop and validate HRM assays, and to assess the genetic variability in a wheat collection. These markers were also used to target meta-quantitative trait loci (MQTL) [71,72] related to drought and heat stresses, as well as yield and yield related traits. Candidate gene analyses were performed for the ISBP markers and the genes were validated by gene expression analyses carried out among different drought and heat stress conditions.

2. Material and methods

2.1. Plant Material and DNA Isolation

Two wheat panels were used to perform the analyses: (i) panel 1: a collection of 62 wheat lines (37 *Triticum aestivum* L., 11 *Triticum turgidum* ssp. *durum* (Desf.) Husn., 11 *Triticum monococcum* L., 2 *Triticum turgidum* ssp *turgidum* L. and one *Triticum urartu* Thumanian ex Gandilyan) from different sources (Supplementary Materials Table S1); (ii) panel 2: a collection of 76 durum wheat (*Triticum turgidum* L) landraces, provided by the Spanish National Plant Genetic Resources Center (CRF-INIA) (Supplementary Materials Table S2). This panel comprised genotypes of three subspecies: 8 *dicoccon* (Schrank) Thell., 21 *T. turgidum*, and 45 *durum* (Desf.) Husn.

Genomic DNA was isolated from young leaf tissue according to the cetyl trimethyl ammonium bromide (CTAB) method of Murray and Thompson [73], as optimized by Hernández et al. [74]. The quality and concentration of samples were assessed by electrophoresis in a 0.8% agarose gel.

2.2. Insertion Site-Based Polymorphism Markers Development

ISBP markers were initially developed based on the wheat chromosome 4A survey sequencing [61] and confirmed in the bread wheat reference genome sequence RefSeq v1 [33].

The assemblies corresponding to the 4A wheat chromosome survey sequencing were generated using the "Newbler v2.7" software package (Roche Diagnostics Corporation, Basel, Switzerland) using default parameters. IsbpFinder [30] was run on the assemblies obtained, and ISBP markers were located on the 4AS and 4AL chromosome arms assemblies. The corresponding 45 ISBP primers were designed using Primer3 (http://primer3.sourceforge.net) and mapped to the bread wheat reference genome RefSeq v1 [33]. Marker set up was carried out using 6 durum and bread wheat lines representative of variability (Supplementary Materials Table S1).

ISBP amplicons obtained by standard PCR (55 °C annealing, [30]) using 5 *T. aestivum* varieties (Supplementary Materials Table S1) were purified by Exonuclease l (Exo I, New England Biolabs, Inc., Ipswich, MA, USA) and SAP treatment (5 µL DNA, 1U Exol, 1xSAP buffer, 1U SAP in 9 µL at 37 °C for 1 h). The purified fragments were then sequenced on an ABI PRISM® 3730XL (Applied Biosystems, Foster City, CA, USA) genetic analyzer using the forward and reverse ISBP primers [30] and using the ABIPRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). HRM analyses were carried out for the same 5 *T. aestivum* varieties (Supplementary Materials Table S1) using 6 ISBP primer pairs previously developed for wheat chromosome 3B [30]: *HRM3B_273339424*, *HRM3B_609364064*, *HRM3B_124761338*, *HRM3B_203288704*, *HRM3B_465802537*, and *HRM3B_331497483*. This analysis was carried out in a Rotorgene™ 6000, model 5-Plex real time PCR (Corbett Research, Mortlake, NSW, Australia). The PCR reaction volume was of 15 µL and the mixture composition was: Master Mix of Type-it HRM PCR Kit (Qiagen, CA, USA); 0.7 µM of each primer and 2 µL of genomic DNA (30 ng). The PCR protocol consisted on an initial denaturation step at 95 °C for 10 min; 45 amplification cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 15 s and a final extension at 72 °C for 20 s; the high-resolution melting was set out by ramping from 65

to 95 °C, with fluorescence data acquisition at 0.1 °C increments (waiting for 2 s every acquisition). HRM results were compared with the amplicon sequencing for the same five samples.

The high-resolution melting analysis was later performed to assign HRM pattern types to the ISBP markers, using 19 durum and bread wheat lines (Supplementary Materials Table S1). The PCR protocol consisted of an initial denaturation step at 95 °C for 5 min; 50 cycles of denaturation at 95 °C for 20 s, annealing at 60 °C for 20 s, and a final extension at 72 °C for 20 s. HRM analysis was undertaken once amplification was completed by ramping from 65 to 95 °C, with fluorescence data acquisition at 0.1 °C increments (waiting for 2 s every acquisition). Results were analyzed by using the Rotorgene software version 1.7 (Qiagen, the Netherlands), and HRM curves were normalized according to the manufacturer's instructions.

2.3. Candidate Genes and Gene Expression Analyses

ISBP markers were mapped in wheat chromosomes 4A and 3B, and then were compared to the wheat MQTLs described in Acuña-Galindo et al. [71]. To obtain the MQTLs positions in wheat chromosomes, marker sequences [71] were extracted from "Graingenes" (graingenes.org) and "Wheat SSR DB markers" (wheatssr.nig.ac.jp), and then located by mapping flanking markers (using BLAST) against RefSeq v1 [33]. Only the markers with a corresponding amplicon shorter than 500 bp and a perfect BLAST match (no gap, no mismatch) were considered.

Markers sequences in chromosomes 4A and 3B were blasted against the RefSeq1 v1 [33] using the parameters "-task", "blastn-short" and "-ungapped". The resulting hits were then processed to pair forward and reverse sequence hits with an amplicon <1000 base pairs (bp). For subsequent analysis, paired sequences were ordered by number of mismatches, so markers position was inferred from the position of pairs with lower number of mismatches (0 in most cases). To identify candidate genes associated to each marker, the results were filtered and the hits with best e-value were selected for each molecular marker. The candidate genes were manually selected within a window of +/−20 kb of the marker's hit in the pseudomolecule [33] gene model annotation. Due to the reduced gene density found for wheat chromosome 3B ISBP markers (only three genes were found), that window was extended to +/−300 kb for this chromosome. Genes described as "uncharacterized protein" were then manually annotated. Sequences were obtained in Ensembl Plants (*T. aestivum* RefSeq v1.1) (https://plants.ensembl.org/), and then searched in UniProt (https://www.uniprot.org/). The annotated hits with e-value 0.0 and a score >2000 were selected, except for the gene *TraesCS4A01G410700*, which possesses a short length (207aa).

To overview the results from gene expression analyses, heatmaps were drawn using the data retrieved from Wheat Expression (www.wheat-expression.com/) and the 'NMF 0.21.0' R package [75]. The information used was generated by Liu et al. [22], Ma et al. [26], and Galvez et al. [27]. Liu et al. [22] experimental seedling samples grown in controlled conditions were associated to NCBI SRA ID SRP045409 (control (IS), heat and drought (PEG induced drought) stress for 1 and 6 h (PEG1 and PEG6), respectively). Ma et al. [26] experimental samples grown in a shelter corresponded to NCBI SRA ID SRP102636 (anther stage irrigated leaf phenotype (AD_C), anther stage drought-stressed leaf phenotype (AD_S), tetrad stage irrigated developing spike phenotype (T_C), and tetrad stage drought-stressed developing spike phenotype (T_S)). Galvez et al. [27] flag leaf samples from field grown plants used have NCBI SRA ID SRP119300 (irrigated (IF), mild stress (MS), and severe stress (SS) flag leaf samples). Transcripts Per Kilobase Millions (TPMs) of genes under every condition were calculated as mean value of TPMs of its constitutive experiments. A differential gene expression (DGE) analysis was performed using RevSeqv1 [33] gene models through two bioinformatic pipelines: Kallisto (version 0.43.0) with the R library "sleuth"(version 0.28.1), and STAR with the R library DESeq2 (version 1.14.1). A consensus threshold for the two pipelines (|lg2FC, β| > 1 and *p*-adjust, Q-value < 0.05 [27]) was used.

2.4. Wheat Variability Assessment

The PCR amplification protocol used was the previously described for HRM pattern type assessment. Samples genotyping was performed using Melt and HRM analysis options of the Rotor-Gene™ 6000 software. PCR was repeated three times to ensure the amplifications. Results were then corroborated with Rotor-Gene™ ScreenClust HRM™ Software. In those cases where the number of genotypes assigned was unclear, ScreenClust HRM™ Software was also used for the final decision. A binary matrix for genotyping results was created and then analyzed using two different software, PhylTools v.1.32 (Wageningen Agricultural University, The Netherlands) and PowerMarker v.3.25 [76]. The first one was used to calculate the genetic distances for haploid data with "individuals" as hierarchy level and the Nei index [77]. PowerMarker was used to obtain the statistics mean allele number, mean gene diversity, and Polymorphism Index Content (PIC). The unweighted pair group method with arithmetic mean (UPGMA) clustering was carried out using the NEIGHBOR module in the Phylip 3.695 package [78] with default parameters. The selected tree method was UPGMA. The final dendrogram was drawn using MEGA v.6.0 [79] software with the results from the genotyping.

The goodness of fit of the UPGMA tree was calculated by the Cophenetic Correlation Coefficient (CCC) using a visual basic program for Microsoft Excel 2000 [80]. The CCC calculated from the linear regression between the corresponding values of the original distance matrix and the cophenetic matrix derived from the calculation of the UPGMA tree.

3. Results

3.1. Markers Sequence Validation and HRM Pattern Types Assignment

To validate markers sequences, difference plots and HRM normalized curves from the high-resolution melting analyses were obtained for each of the six ISBP markers for wheat chromosome 3B and compared with their corresponding amplicon sequences. The HRM profiles were successfully validated. Sequence polymorphism from one to five nucleotides were detected by HRM analyses. Those included both transitions (examples are shown in Figure 1a,c,f), transversions (Figure 1e) and both (Figure 1b,d).

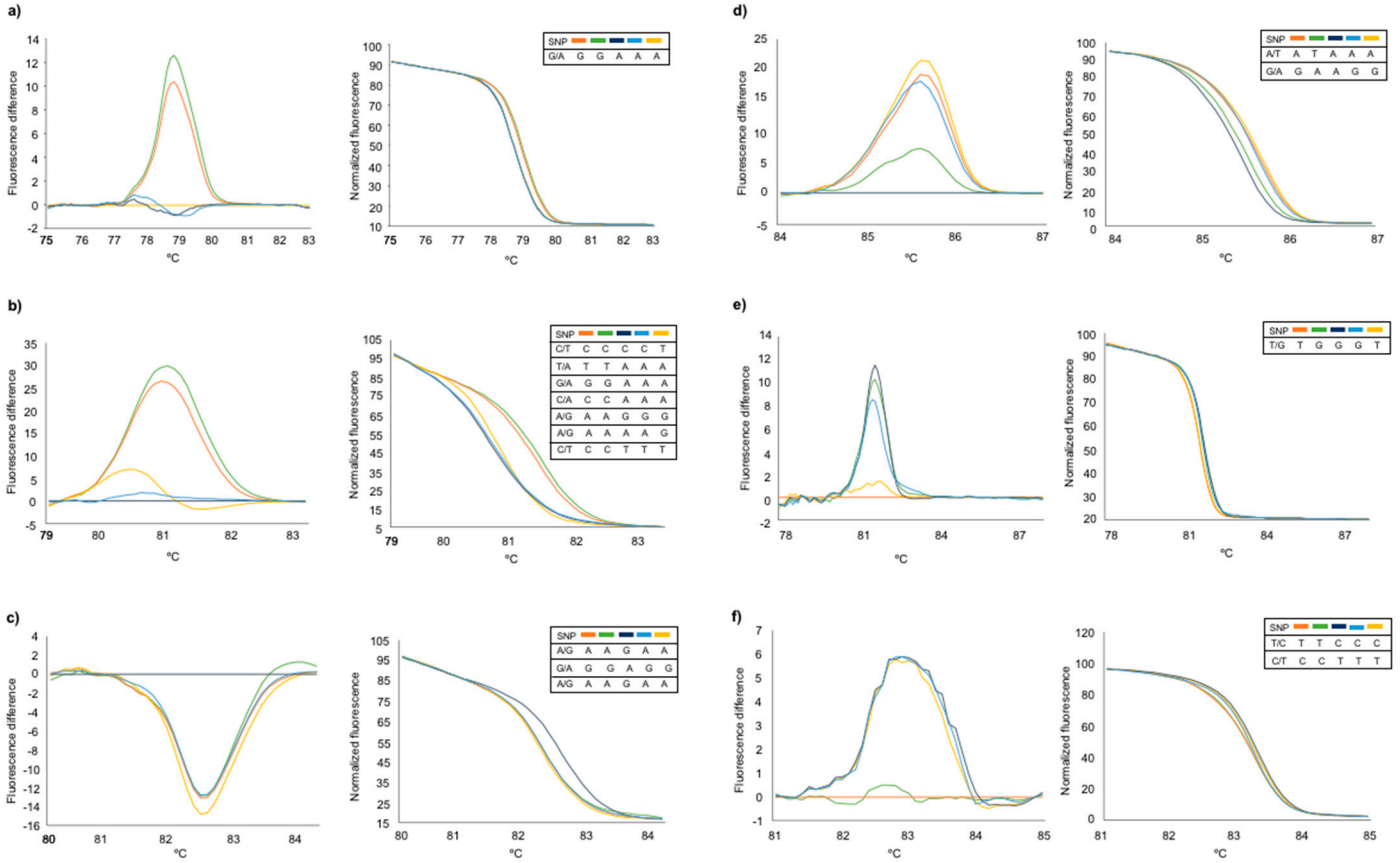

Figure 1. Sequence validation for insertion site-based polymorphisms (ISBP) markers using high-resolution melting (HRM) analysis. Normalized curves (on the right) and difference plots (on the left) are shown for 6 ISBP markers for wheat chromosome 3B, with different nucleotide variations: (**a**) marker *HRM3B_273339424*—single nucleotide transition (A/G); (**b**) marker *HRM3B_124761338*—five nucleotide variations, three transitions (C/T, G/A, and A/G) and two transversions (T/A and C/A); (**c**) marker *HRM3B_203288704*—three nucleotide transitions (A/G, G/A, and A/G); (**d**) marker *HRM3B_465802537*—two nucleotide variations, one transversion (A/T) and one transition (G/A); (**e**) marker *HRM3B_609364064*—one transversion (T/G); and (**f**) marker *HRM3B_331497483*—two transitions (T/C and C/T).

The HRM pattern type assignment was based on the pattern of normalized high-resolution melting curves obtained, and their potential to genotype a high number of varieties. The ISBP HRM patterns were classified into four different types (Table 1, Figure 2): (i) pattern type A, excellent markers to genotype a high number of wheat varieties simultaneously. The HRM curves are very different and easily distinguishable into classes (Figure 2a); (ii) pattern type B, good markers to differentiate several groups of varieties (genotypes) in the same run. HRM curves can be differentiated in an easy way (Figure 2b); (iii) pattern type C, good HRM markers, but not recommended for genotyping a broad variety of samples. The differentiation between HRM curves is not clear in all cases (Figure 2c); and (iv) pattern type D, assigned to markers which are not recommended for HRM genotyping, due to a low amplification efficiency or to a gradient of HRM curves that hinders precise classification into classes (Figure 2d). Twelve ISBP markers were classified as HRM pattern type A; 4 markers showed HRM pattern type B; 10 markers had HRM pattern type C; and 13 were considered with pattern type D (Table 1). Four of the 45 wheat chromosome 4A ISBP primer pairs designed (Table 2), were discarded for the rest of analyses due to non-reliable PCR amplifications.

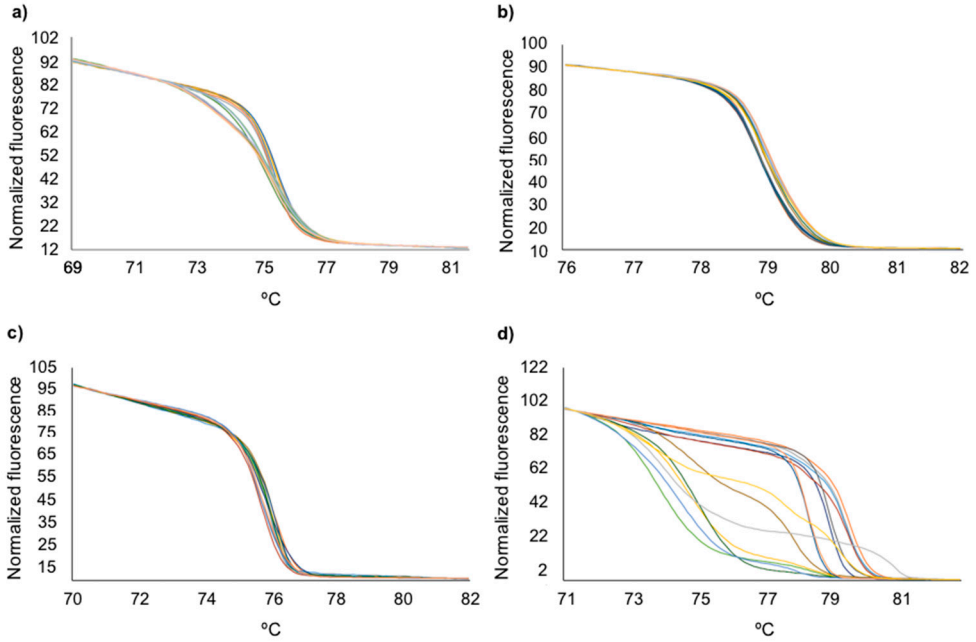

Figure 2. High resolution melting pattern types assessment for wheat chromosome 4A ISBP markers. Normalized HRM curves for 19 samples were shown for (**a**) marker *HRM4A_67413676* (pattern type A); (**b**) marker *HRM4A_618105078* (pattern type B); (**c**) marker *HRM4A_317085557* (pattern type C); and (**d**) marker *HRM4A_291420130* (pattern type D).

Table 1. Insertion site-based polymorphism markers developed for wheat chromosome 4A. ISBP primers selected for the wheat collection variability assessment are shown in bold.

Marker ID	Chr	Pattern	Forward Primer (5′→3′)	Reverse Primer (5′→3′)	Amp. Size
HRM4A_2791416 *	4AS	A	TCCTACAAAAACGTCTTATATTTTGG	GATCACTTGCACGTTGCATT	103
HRM4A_9618320	4AS	C	CGTCAGCTCAAAGGAAAACC	GGGAGGAAATTTGCGAGTC	151
HRM4A_73613394	4AS	D	CGGTCCTTGTGATGATGTTG	CTTTGTAGGCCCCATCTGAA	136
HRM4A_36371442	4AS	D	GCATGTGGTCATGTTCTTGG	TCAAAAACGCTTTTATATTATGGGA	193
HRM4A_38654555 *	4AS	A	TCTGAAAGAACCTCAGCTTATTACTT	TTTGGTAAAATTGAGGGACCA	101
HRM4A_47108251	4AS	n/s	ACACGTGGGAGATATAGCCG	AAACCCTAGGTCCCACTGCT	168
HRM4A_60660613	4AS	C	CATCCTCTCAGGCCATGAAT	CAAAATTCAAACTTTTCGGTTTC	141
HRM4A_U-1	-	n/s	AGAGCGCTTAAGTTTGGCTG	TCACTCATTTCAGTCCGGAAG	161
HRM4A_56502024	4AS	D	CAAACGATGCTCCTCTGTCA	TCCATCTATCTGTATTTCGTATTGAA	119
HRM4A_67413676	4AS	A	CGAGTCCTAGCGAGTTCCTG	AAAAACATTGCATAAATGGATGG	109
HRM4A_59280888	4AS	B	CATCCACATGGATTTTGCAG	CGATTTGGTACGCTAGGAGG	207
HRM4A_141912346	4AS	A	AGCACAAGCATGCAATGAAG	TTATCTCGTGTAGGACCGGG	143
HRM4A_99034796	4AS	n/s	CCTCCGTTCGGAATTACTTG	TTCATGCAGCAGCAGTTACC	157
HRM4A_109848074 *	4AS	A	ATGAGACTTTTGACGACCGC	CAAGCTTTTTCAGACGGAGG	139
HRM4A_141111880	4AS	D	AGATCTGCTGGACATAAGCACA	CCCTCCGTCCCAAAATAAGT	117
HRM4A_162423177	4AS	C	AAGCAAGAAGCGAAAACAGC	AATCATCTAGTCGGTTGCGG	180
HRM4A_176868209	4AS	C	TTCCGAATTACTTGTCTCGGAT	CACAGGCTCGGATAGGCTAC	191
HRM4A_183985221	4AS	C	TCCAGAAAATCTGTAGGCACTG	AGATGGACGCGATAAGATGG	108
HRM4A_149049302	4AS	D	CCTCCGTTCGGAATTACTTG	ATGAAAGGCAGGCTAGACCA	223
HRM4A_617938526	4AL	C	CTTCGTCCTTCCTCGCATAG	GTAAGGTAGTGATCTAAACGGTGTT	113
HRM4A_618105078	4AL	B	AGTCATGGCACCAACAACAA	AGAGTTGCCGTGCCACTTAT	162
HRM4A_U-2	-	n/s	TTTCTAAGGGGTAAGGGCGT	TAGAGGGTTGTGCTGGTTCC	100
HRM4A_716986193 *	4AL	A	CTGCACCATAGATCGAAGCA	ACAGGACAATTGGAGACCCA	159
HRM4A_U-3	-	A	TTGAACTGCCAAAAACGTCTCA	CTCCCTCCTATAACCACCATTG	100
HRM4A_548541053	4AL	D	CGGTGCTAGATACATCCGTTT	AACCAAGTAAGCATGTACTAGAGAAAA	112
HRM4A_714743756	4AL	A	CGCTAGTATAGTGTCAAAAACGC	ATGTAGGATGTCCCTGCGTC	117
HRM4A_U-4 *	-	A	CAGACAATGTGCAAAACAACC	AAAAGAGTTCATGTACAAGGGGA	116
HRM4A_U-5	-	n/s	TCCACCTTATAAACACCCGC	TGTATTTCCAGGACGGAAGG	202
HRM4A_460238681	4AL	C	GGTCTGTATTGAAATCTCTAAAAGTGC	CCTTTTGTTCAGCCTGTGGT	119
HRM4A_583704598 *	4AL	A	CCCTCTGTAAACTAATATAGAATGCG	CTTGTTCCCTCTGCTCCTTG	165
HRM4A_317085557	4AS	C	ACATGGGTGACCCTATCCAA	CGGACTGGTCCATTAGGGTT	162
HRM4A_291420130	4AS	D	AAGTGGAAAAGGCACAATGC	CAAACACTTTGCCAACATGG	183
HRM4A_681664894	4AL	D	TATGCTTGAGTGCTTGGCTG	CATCCATTTGAGCGACAACT	121

Table 1. *Cont.*

Marker ID	Chr	Pattern	Forward Primer (5′→3′)	Reverse Primer (5′→3′)	Amp. Size
HRM4A_683608822	4AL	C	TCAGTTTTAGGTCCCGTTGC	TGACACTACTCTAAGTTACTCCCCA	196
HRM4A_U-6	-	D	TTCCAAGAAAATGTTCGCAA	TCCTTCGTTCAAGACTCGCT	177
HRM4A_U-7	-	B	TCCCTAGCTGATGATTTGGG	ATAATAGCTCCATACGCGCC	107
HRM4A_660524139 *	4AL	A	GATAAATCTAAGATAAGCTTTTTGGG	GGGACACAATGTGATGGTGA	116
HRM4A_U-8	-	C	GGCCCTAGAAATGCAAATGA	TTCCCACCTCAATAACTGGG	127
HRM4A_U-9	-	B	TCTTCACTCGTTTCAGTCCG	AGACGAGCACACACGCATAC	131
HRM4A_702156718	4AL	A	CCTTTGGCAACAACACAATG	ATTGGCAGATTCTTCAAGCG	133
HRM4A_U-10	-	D	AGCCGAGGAAGGTTCACATA	TCCATTTATAAACAAATATAAGAGCG	119
HRM4A_U-11	-	A	CGGTCAATGTATACTACCGTCG	ATCGGGAACCACCAATGTTA	164
HRM4A_U-12	-	D	TGTTTGCTGAGGACCAACAG	CCGGGGGTAATCCTAATTTT	111
HRM4A_U-13	-	D	ATTGTTCGTTCCGTTTTTGG	ACTCCCTCCGTCCCATAATA	114
HRM4A_U-14	-	D	CCCTCTGTAAAGAAATATAAGACCGT	TGGATGCAGCTAACTCGAAA	107

Chr: chromosome location; Pattern: high resolution melting pattern type (**A**: excellent marker to genotype different wheat varieties simultaneously; **B**: good marker to genotype several wheat varieties simultaneously; **C**: good marker but not recommended for a broad variety of wheat samples; **D**: marker not recommended for HRM genotyping; and **n/s** for markers non-suitable for HRM); Amp. size: amplicon size in base pairs (bp); *: ISBP markers used in the durum wheat collection variability assessment.

Table 2. Wheat chromosome 4A ISBP markers linked to genes which alter their expression in response to water stress treatments. DE genes are shown in bold. The positive and negative values in "*Dist*" column indicate if the corresponding gene is downstream or upstream of the marker.

Marker ID	Chr	Dist (bp)	Gene ID	Description	Sum of TPMs
HRM4A_2791416	4A	−814	TraesCS4A01G003600	Alpha/beta-Hydrolases superfamily protein	8.79
		−5141	**TraesCS4A01G003500**	Thionin-like protein	124.0
HRM4A_36371442	4A	−2736	**TraesCS4A01G043500**	STAS domain-containing protein	47.32
HRM4A_38654555	4A	−9961	TraesCS4A01G047100	Activating signal cointegrator 1 complex subunit 2	12.70
		−14,885	**TraesCS4A01G047000**	Formin-like protein	4.87
HRM4A_56502024	4A	−417	TraesCS4A01G060200	BHLH domain-containing protein	10.54
		−5039	TraesCS4A01G060100	Uncharacterized protein	50.64
HRM4A_67413676	4A	975	**TraesCS4A01G069200**	Armadillo repeat only	21.26
HRM4A_99034796	4A	−5609	TraesCS4A01G092400	SH3 domain-containing protein	43.46
HRM4A_109848074	4A	97	TraesCS4A01G098300	Xylosyltransferase 1	21.59
HRM4A_141111880	4A	−3308	TraesCS4A01G116200	MYND-type domain-containing protein	50.62
HRM4A_162423177	4A	9	TraesCS4A01G126300	Transcription factor TGA2.2 *	83.14
HRM4A_176868209	4A	−406	TraesCS4A01G132000	Uncharacterized protein	17.27
HRM4A_183985221	4A	−5474	TraesCS4A01G134900	Lung seven transmembrane receptor family protein, expressed *	44.02

Table 2. *Cont.*

Marker ID	Chr	Dist (bp)	Gene ID	Description	Sum of TPMs
HRM4A_317085557	4A	−14,884	TraesCS4A01G157000	SAP domain-containing protein	127.49
HRM4A_460238681	4A	−9041	TraesCS4A01G182900	SPRING type domain-containing protein *	11.42
HRM4A_548541053	4A	−11,850	TraesCS4A01G239500	ATP-dependent RNA helicase dhx8 *	19.65
HRM4A_583704598	4A	3305	TraesCS4A01G271900	Histone-lysine N-methyltransferase	11.28
HRM4A_617938526	4A	−5243	TraesCS4A01G335200	Protein kinase domain-containing protein *	10.09
HRM4A_681664894	4A	−3025	TraesCS4A01G408900	Protein PIR *	33.28
HRM4A_683608822	4A	−2685	**TraesCS4A01G410700**	Ras-related protein RABC2a *	82.16
HRM4A_716986193	4A	−3576	TraesCS4A01G671200LC	Peptidase M20/M25/M40 family protein	104.94
HRM4A_U-2	4A	−9642	TraesCS4A01G287200	C2 calcium/lipid-binding and GRAM domain containing protein	2.72

Chr: chromosome location; Dist (bp): distance from the gene to the marker in base pairs (bp); TPMs: Transcripts Per Kilobase Millions; and *: genes manually annotated.

3.2. Candidate Gene Analysis

After mapping ISBP markers and comparing them with the MQTLs positions previously described in Acuña-Galindo et al. [71], some markers for the wheat chromosome 4A were found in the proximity of interesting QTLs or within MQTLs related to drought and heat stress tolerance, as well as QTLs for yield components (Figure 3a). Two ISBP markers, *HRM4A_317085557* and *HRM4A_460238681*, were located within MQTL30 [71], related to the physiological drought trait and root vigor. Markers *HRM4A_617938526* and *HRM4A_618105078* were placed in MQTL31 [71], in proximity to QTLs related to drought and heat stresses. The marker *HRM4A_660524139* was placed close to two of the QTLs located within MQTL31, associated to traits for yield component and coleoptile vigor, and also heat stress. Marker *HRM4A_583704598* was placed between MQTL30 and MQTL31, in proximity to 2 QTL related to drought and heat stresses. Finally, markers *HRM4A_681664894* and *HRM4A_683608822* were placed in MQTL32 [71], close to QTLs related to drought; and markers *HRM4A_702156718*, *HRM4A_714743756*, and *HRM4A_716986193* were found close to a QTL located in MQTL32, related to drought tolerance (Figure 3a).

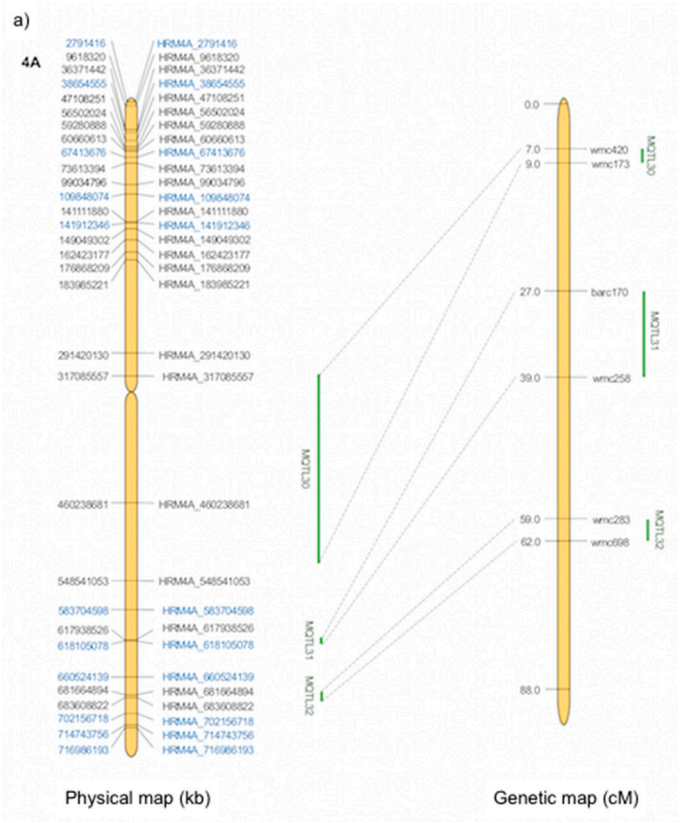

Figure 3. *Cont.*

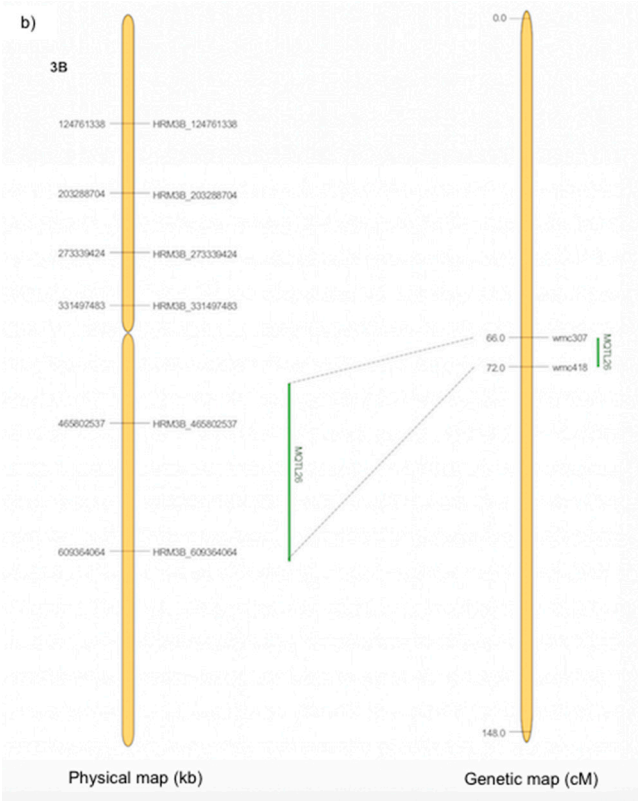

Figure 3. Physical and genetic maps for markers located in chromosome 4A (**a**) and 3B (**b**) including location for meta-quantitative trait loci (MQTLs) [71] associated to heat and drought stresses tolerance. Markers used in variability assessment are shown in blue color. MQTLs are indicated using green lines.

Two of the wheat chromosome 3B ISBP markers (*HRM3B_465802537* and *HRM3B_609364064)* were mapped within MQTL26 [71], in proximity to QTLs controlling yield component and biomass traits, and also related to heat stress (Figure 3b).

Additionally, as result of the candidate gene analysis, the developed ISBP markers for wheat chromosome 4A mapped in the proximity of 61 genes (Supplementary Materials Table S3 and Figure S1). The chromosome position for the ISBP and their closest genes are shown in Figure 4a.

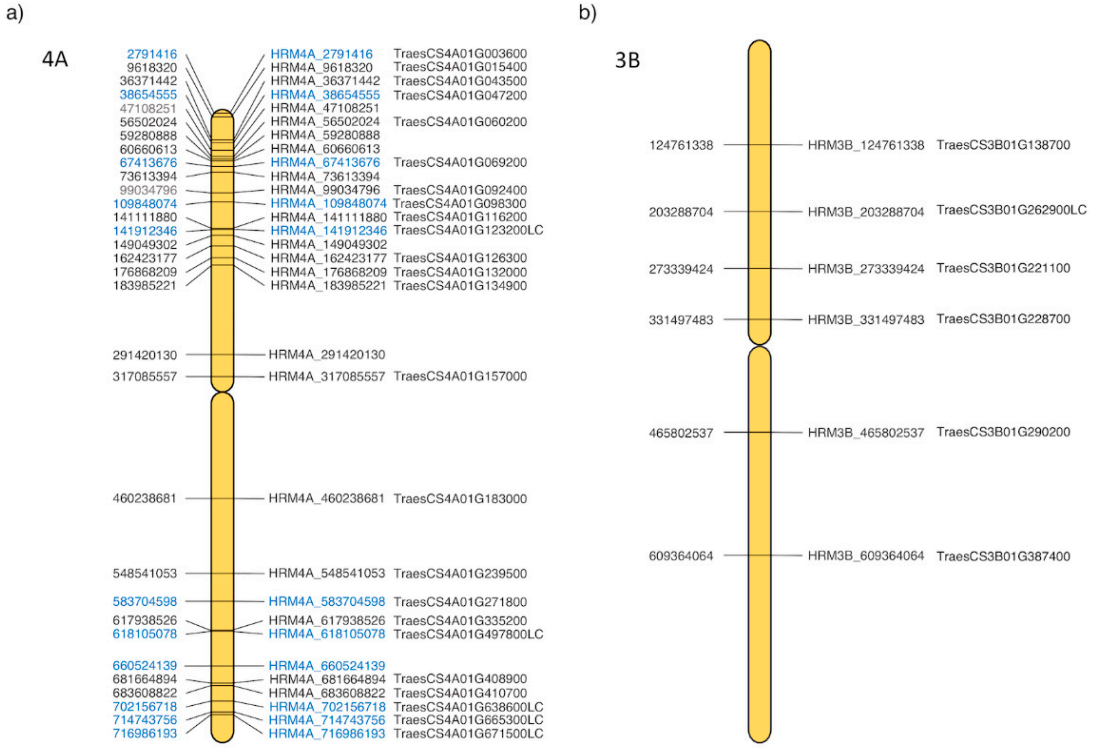

Figure 4. Physical maps for the wheat chromosomes 4A (**a**) and 3B (**b**) showing the molecular markers location and their nearest genes found within a window of +/−20 kb and +/−300 kb, respectively. ISBP markers used in wheat variability assessment are highlighted in blue.

Based on gene expression differences under different drought stress conditions, we filtered 23 genes (37.7% of the total genes) with a TPM value above 2.5 (Table 2 and Figure 5). An expression heatmap, using all available public studies RNASeq data in wheat drought responses [22,26,27] is shown in Figure 5.

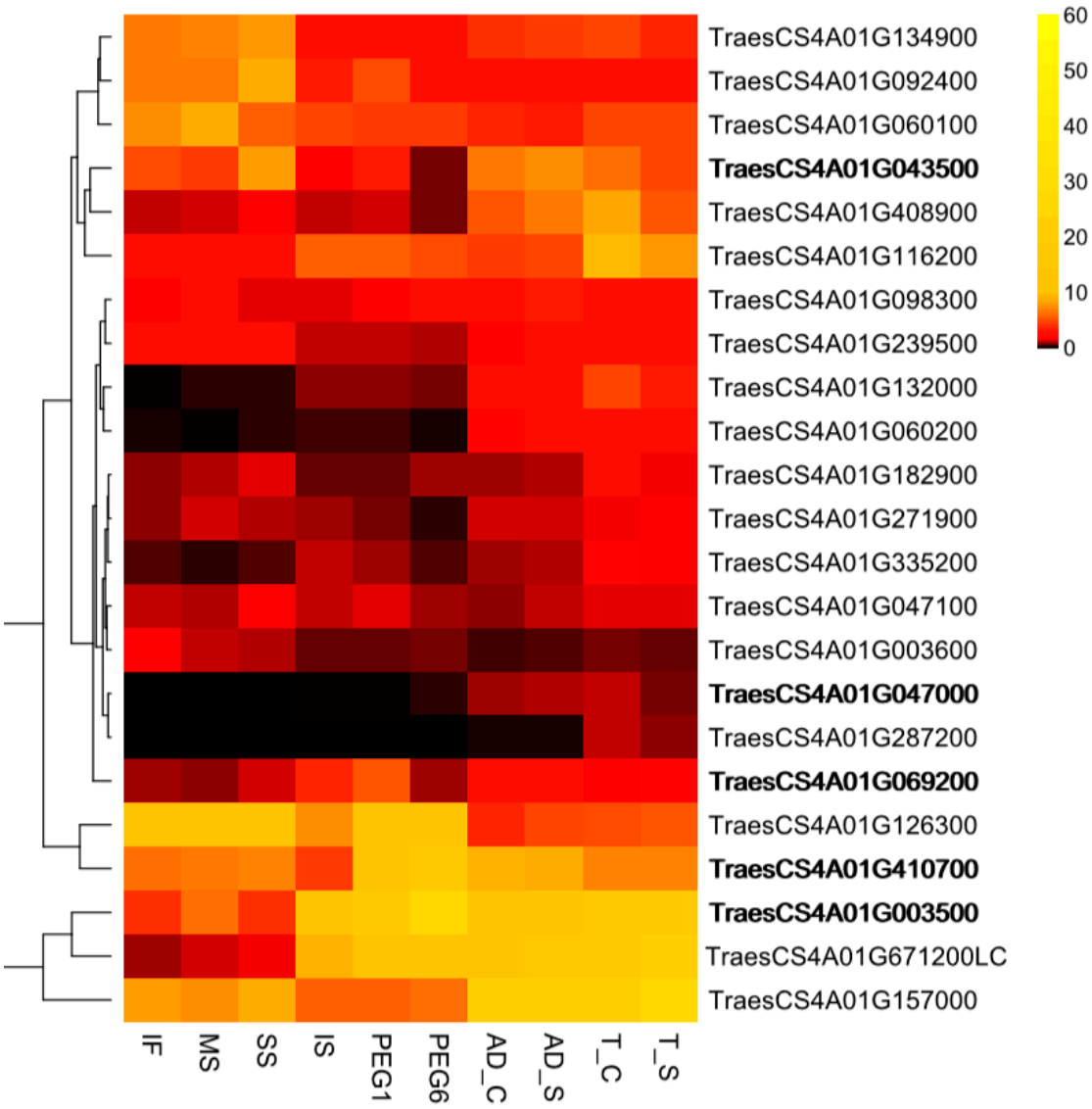

Figure 5. Gene expression analysis under different water stress conditions for candidate genes located within a window of +/−20 kb to the wheat chromosome 4A ISBP markers. Differentially expressed genes (DEGs) are shown in bold. IF: irrigated field conditions; MS: mild stress field condition; SS: severe stress field condition [27]; IS: seedling control; PEG1: seedling 1 h PEG stress; PEG6: seedling 6 h PEG stress [22]; AD_C: anther stage irrigated shelter phenotype; AD_S: anther stage drought stressed shelter phenotype; T_C: tetrad stage irrigated shelter phenotype; and T_S: tetrad stage drought shelter phenotype [26].

The wheat chromosome 3B ISBP markers mapped in the proximity of 49 genes (Supplementary Materials Table S4 and Figure S2). The closest genes are shown next to the corresponding marker in Figure 4b. Seventeen of these genes (34.69% of the total) showed TPM values above 2.5 (Table 3 and Figure 6). The drought responsive genes mapped by ISBP markers located in proximity or within QTLs or MQTLs are shown in Table 4.

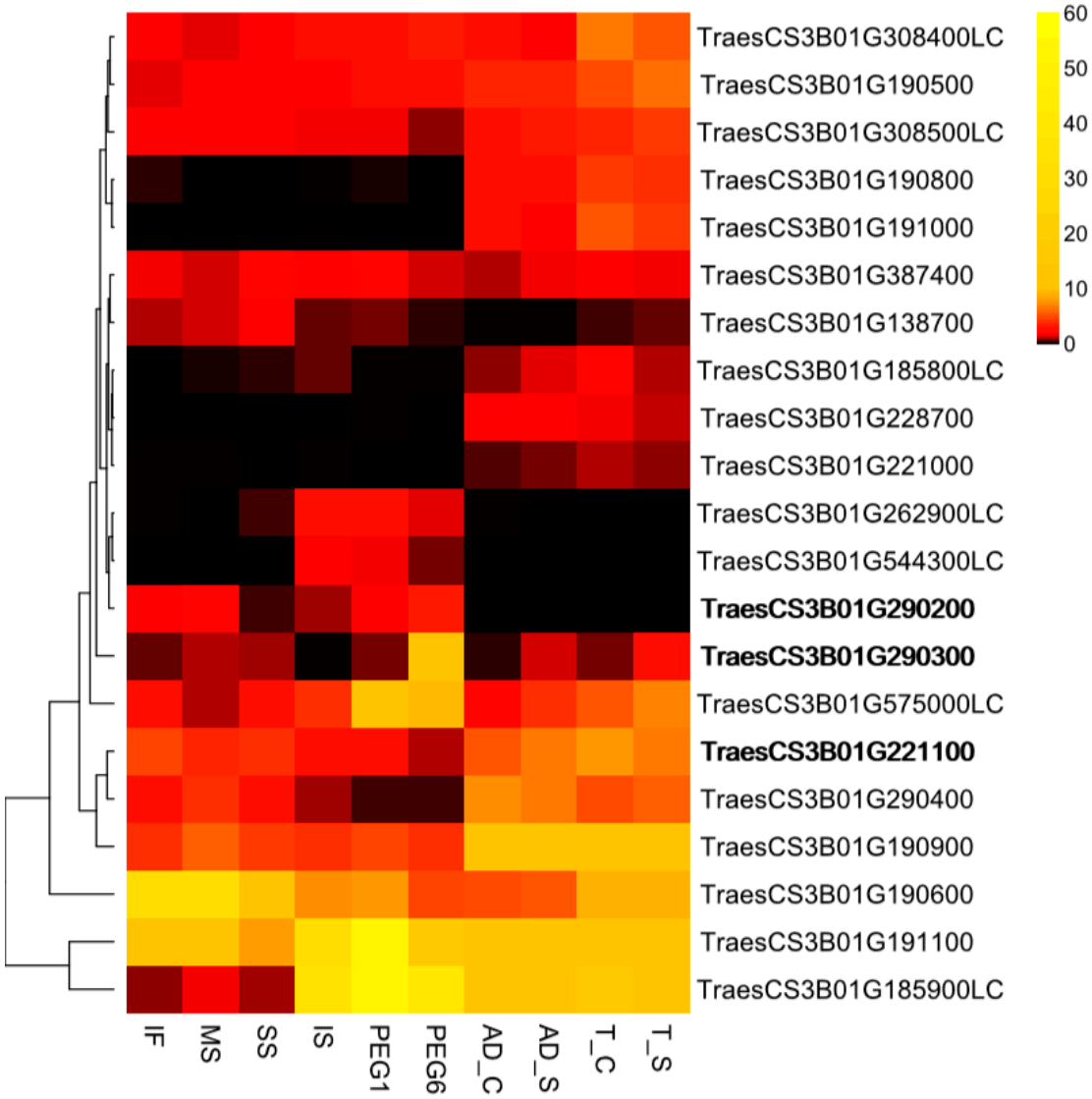

Figure 6. Gene expression analysis under different stress conditions for candidate genes located within a +/−300 kb window and in proximity to wheat chromosome 3B ISBP markers. Differentially expressed genes (DEGs) are shown in bold. IF: irrigated field conditions; MS: mild stress field conditions; SS: severe stress field conditions [27]; IS: seedling PEG shock control; PEG1: seedling 1 h PEG stress; PEG6: seedling 6 h PEG stress [22]; AD_C: anther stage irrigated shelter phenotype; AD_S: anther stage drought stressed shelter phenotype; T_C: tetrad stage irrigated shelter phenotype; and T_S: tetrad stage drought shelter phenotype [26].

Table 3. Wheat chromosome 3B ISBP markers linked to genes which alter their expression in response to water stress treatments. DE genes are shown in bold. The positive and negative values in "*Dist*" column indicate if the corresponding gene is downstream or upstream of the corresponding marker.

Marker ID	Chr	Dist (bp)	Gene ID	Description	Sum of TPMs
HRM3B_124761338	3B	6356	TraesCS3B01G138700	Ribonucleoside-diphosphate reductase	7.48
		8268	TraesCS3B01G185800LC	Serine/threonine-protein phosphatase 6 regulatory subunit 1	7.25
		14,040	TraesCS3B01G185900LC	LEM3 (Ligand-effect modulator 3)-like	180.07
HRM3B_203288704	3B	227,619	TraesCS3B01G262900LC	Retrovirus-related Pol polyprotein LINE-1	7.05
		260,457	TraesCS3B01G190500	Polynucleotidyl transferase ribonuclease H-like superfamily protein	28.66
		266,554	TraesCS3B01G190600	Beta-amylase	118.13
HRM3B_273339424	3B	−89,674	**TraesCS3B01G221100**	Protein kinase superfamily protein	43.92
		−90,463	TraesCS3B01G308500LC	translation initiation factor 3 subunit H1	22.30
		−101,322	TraesCS3B01G221000	Kelch repeat protein, putative	4.08
		−286,319	TraesCS3B01G308400LC	Ribonuclease H-like superfamily protein	29.01
HRM3B_331497483	3B	55,223	TraesCS3B01G228700	carboxyl-terminal peptidase, putative (DUF239)	6.57
HRM3B_465802537	3B	83,072	**TraesCS3B01G290200**	Glycosyltransferase	9.98
		125,805	**TraesCS3B01G290300**	ABC transporter B family protein	19.57
		296,991	TraesCS3B01G290400	zein-binding protein (Protein of unknown function, DUF593)	34.58
HRM3B_609364064	3B	−24,580	TraesCS3B01G387400	SAC3/GANP/Nin1/mts3/eIF-3 p25 family protein	15.46
		−31,936	TraesCS3B01G575000LC	Myosin-1	48.25

Chr: chromosome location; Dist (bp): distance from the gene to the marker in base pairs (bp); and TPMs: Transcripts Per Kilobase Millions.

Table 4. Drought responsive candidate genes tagged by the developed HRM-ISBP markers. DE genes are shown in bold and differentially expressed genes are indicated with "*". Gene expression responses to drought treatments are shown in Figures 5 and 6, and Tables 3 and 4. The positive and negative "Dist" column values indicate if the corresponding gene is downstream or upstream of the marker.

Marker ID	Chr	MQTL	Dist (bp)	Gene ID	Description
HRM4A_716986193	4A	32	−3576	**TraesCS4A01G671200LC**	Peptidase M20/M25/M40 family protein
HRM4A_714743756	4A	close to QTL in MQTL32	−5397	TraesCS4A01G665300LC	LINE-1 reverse transcriptase-like protein
			−6886	TraesCS4A01G665200LC	C4-dicarboxylate transport protein
			−12,058	TraesCS4A01G665100LC	NBS-LRR disease resistance protein
F			−14,064	TraesCS4A01G665000LC	Disease resistance protein (NBS-LRR class) family
			14,217	TraesCS4A01G665400LC	NBS-LRR disease resistance protein
			17,933	TraesCS4A01G665500LC	Transposase

Table 4. *Cont.*

Marker ID	Chr	MQTL	Dist (bp)	Gene ID	Description
HRM4A_583704598	4A	between MQTL30 and 31	−2715	TraesCS4A01G271800	Kinase family protein
			3305	**TraesCS4A01G271900**	Histone-lysine N-methyltransferase
			−6092	TraesCS4A01G431700LC	Serine/threonine-protein kinase mph1
HRM_4A660524139	4A	close to QTL in MQTL31			
HRM4A_702156718	4A	close to QTL in MQTL32	−1999	TraesCS4A01G638600LC	Retrotransposon protein, putative, unclassified
			14,448	TraesCS4A01G638700LC	Kinase, putative
HRM4A_618105078	4A	31	−2442	TraesCS4A01G497800LC	Receptor-like protein kinase
			−11,813	TraesCS4A01G497700LC	Protein FAR1-RELATED SEQUENCE 5
			16,466	TraesCS4A01G335600	NBS-LRR-like resistance protein
			−19,471	TraesCS4A01G335500	NBS-LRR disease resistance protein
HRM4A_617938526	4A	31	−5243	**TraesCS4A01G335200**	Protein kinase domain-containing protein *
HRM4A_460238681	4A	30	132	TraesCS4A01G183000	Protein kinase domain-containing protein
			−9041	**TraesCS4A01G182900**	SP-RING-type domain-containing protein *
HRM4A_317085557	4A	30	−14,884	**TraesCS4A01G157000**	SAP domain-containing protein
HRM4A_683608822	4A	32	−2685	**TraesCS4A01G410700** *	Ras-related protein RABC2a *
HRM3B_609364064	3B	26	52,100	TraesCS3B01G575100LC	Transposon protein, putative, CACTA, En/Spm sub-class
			−24,580	**TraesCS3B01G387400**	SAC3/GANP/Nin1/mts3/eIF-3 p25 family protein
			−31,936	**TraesCS3B01G575000LC**	Myosin-1
			−53,463	TraesCS3B01G574900LC	Endonuclease/exonuclease/phosphatase family protein
			−59,628	TraesCS3B01G387300	PP2A regulatory subunit TAP46
HRM3B_465802537	3B	26	83,072	**TraesCS3B01G290200** *	Glycosyltransferase
			−88,899	TraesCS3B01G451200LC	phospholipase-like protein (PEARLI 4) family protein
			−95,341	TraesCS3B01G451100LC	Retrotransposon protein, putative, unclassified
			−98,315	TraesCS3B01G451000LC	Ubiquilin-1
			−106,901	TraesCS3B01G290100	Ribose-5-phosphate isomerase A
			125,805	**TraesCS3B01G290300** *	ABC transporter B family protein
			138,676	TraesCS3B01G451300LC	Polynucleotidyl transferase, ribonuclease H-like superfamily protein
			−144,473	TraesCS3B01G450900LC	Protein regulator of cytokinesis 1
			−147,938	TraesCS3B01G450800LC	Beta-hexosaminidase
			161,237	TraesCS3B01G451400LC	Endonuclease/exonuclease/phosphatase family protein
			296,991	**TraesCS3B01G290400**	zein-binding protein (Protein of unknown function, DUF593)
			297,913	TraesCS3B01G290500	Potassium channel

Chr: chromosome location; MQTL: meta-quantitative trait loci where the marker was located; and Dist (bp): distance from the gene to the marker in base pairs (bp).

After differential gene expression analysis we obtained 5 DE genes in chromosome 4A and 3 in chromosome 3B, which were up and down-regulated by the PEG drought treatment [22]. The 4A chromosome DE genes *TraesCS4A01G003500* and *TraesCS4A01G043500,* were up and down regulated under PEG6 drought treatment, respectively. Genes *TraesCS4A01G047000, TraesCS4A01G410700* were up-regulated, while the gene *TraesCS4A01G069200* was down-regulated under PEG6 treatment. Chromosome 3B gene *TraesCS3B01G221100* was down-regulated under PEG6 treatment, while genes *TraesCS3B01G290200* and *TraesCS3b01G290300* were up-regulated (Supplementary Materials Table S5).

3.3. Wheat Variability Assessment by High Resolution Melting Analysis

To assess the polymorphism levels in a wheat collection, the 45 ISBP markers developed for the wheat chromosome 4A (Table 1), were evaluated. Thirteen of them were selected based on their reproducibility and polymorphism (Table 1) and were used in HRM analyses to study the genetic diversity among durum and bread wheat lines in panel 1 (Supplementary Materials Table S1).

The number of alleles detected for these markers ranged from 2 to 7 (mean = 3.38), and the polymorphism index content varied between 0.24 and 0.68 (mean = 0.52) (Table 5).

Table 5. Genetic parameters for the wheat chromosome 4A ISBP markers used in the variability assessment.

Marker ID	HRM Type	No. of Alleles	PIC
HRM4A_2791416	A	2	0.336
HRM4A_38654555	A	2	0.566
HRM4A_67413676	A	3	0.584
HRM4A_141912346	A	5 *	0.595
HRM4A_109848074	A	2	0.544
HRM4A_618105078	B	4 *	0.677
HRM4A_716986193	C	4	0.602
HRM4A_U-3	C	2 *	0.242
HRM4A_714743756	A	2 *	0.368
HRM4A_U-4	A	3 *	0.474
HRM4A_583704598	A	7 *	0.571
HRM4A_660524139	A	4 *	0.57
HRM4A_702156718	A	4 *	0.563
Mean		3.38	0.515

HRM type: high resolution melting pattern type; PIC: polymorphism index content; *: one of the alleles was described as "null genotype".

The cluster analysis shows three clearly differentiated clusters (Figure 7). The first one contains 30 *T. aestivum* lines, while the remaining 8 bread wheat lines (TaesIN-13, TaesLI-06, TaesLI-07, TaesIF-06, TaesIF-08, TaesIF-07, TaesLI-05, and TspeBO-01) were placed within the second cluster. This cluster also contains *Triticum durum* and *Triticum dicoccoides* accessions, while *Triticum monococcum* and *Triticum urartu* were placed in the third cluster (Figure 7). The cophenetic correlation coefficient obtained for the UPGMA tree was 0.86.

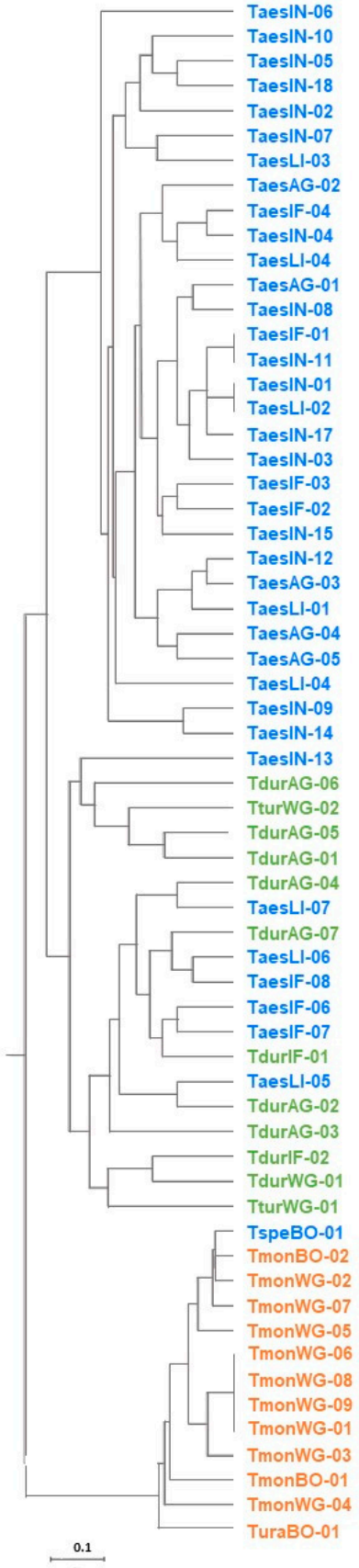

Figure 7. Phylogenetic unweighted pair group method with arithmetic mean (UPGMA) tree showing the relationships among 62 wheat lines genotyped with 13 ISBP markers. *Triticum aestivum* lines are shown in in blue, *T. durum* and *T. dicoccoides* in green, and *T. monococcum* in orange.

Seven of these ISBP markers (Table 1) selected by their efficiency and polymorphism for durum wheat, were used for the variability assessment of durum wheat lines in panel 2 (Supplementary Materials Table S2). The UPGMA tree for the wheat panel 2 is shown in Supplementary Materials Figure S3a,b. This cluster analysis resulted in 9 differentiated clusters. Five lines (BGE002866, BGE013055, BGE020464, BGE013652, and BGE013722) were not placed in any of these clusters. The observed distribution of durum wheat lines across the clusters could be associated in some cases to the geographical location and agroclimatic areas (Supplementary Materials Figure S3c). There are two clear clusters (clusters 4 and 6) where species from southern Spain are presented in a larger proportion. Furthermore, wheat lines placed in cluster 1 are mainly located in norther temperate zones without dry season and temperate summer; while cluster 4 contains landraces mainly located in southern template areas with dry and cool summer. The CCC obtained for the UPGMA tree was 0.67. The number of alleles detected in this analysis ranged from 3 to 6 (mean allele number = 3.86), and the PIC mean value was 0.486 (Supplementary Materials Table S6).

4. Discussion

Insertion site-based polymorphism markers have been described as a useful tool for wheat genomic studies [35] and attractive alternative to markers as SSR or SNP, due to the high repetitive content of some cereal genomes [81,82]. Due to their straightforward design and high polymorphism, there are previous studies which developed and used specific ISBP markers for several wheat chromosomes and different purposes (i.e., Barabaschi et al. [83] for the bread wheat chromosome 5A, focused on polymorphism assessment; Lucas et al. [84], who used markers derived from the wheat chromosome 1A to map this chromosome, and also with marker assisted breeding purposes; Li et al. [36] applied wheat chromosome 3B ISBP linked to mildew resistance genes in durum wheat; or Sehgal et al. [41], who used chromosome 3A ISBP markers for gene discovery and physical mapping). In this work, new ISBP markers were developed for the wheat chromosome 4A, which contains interesting genes related to biotic and abiotic stresses, as drought tolerance [65,68,69]. It also harbors loci related to essential agronomic traits as yield and grain quality [62,64,66]. The ISBP markers resulted highly polymorphic (Table 5). Thirteen of the developed ISBP markers were used for wheat variability assessment and showed melting curve polymorphisms, seven of them with a PIC value higher than 0.50. Thus, they resulted highly resolutive tools for wheat variability assessment using a cost-effective technique as high resolution melting analysis (HRM). This technique is described as an optimized methodology for melting curve assessment, which allows the determination of melting temperature and profile of an amplicon [85]. Some studies have highlighted some advantages for the HRM technique, as its reduced cost per sample in comparison with other techniques used for SNP detection [86–88]. It is worth noting that the required system consists of standard and affordable RT-PCR equipment, which is suitable for in-house genotyping and adequate for small/medium breeders. Other advantages for HRM are the excellent results for the detection of homozygous and heterozygous variants [46,89–91]; its use for gene mapping, SNPs and mutations [46,90,92]; and its efficiency for the identification of species and closely related varieties [88,93–97]. In this regard, our results from ISBP markers sequence validation, as well as HRM genotyping, support the efficiency of HRM analysis in wheat varieties differentiation. Our results are in agreement with Dong et al. [53], who highlighted that HRM does not require any digestion or gel electrophoresis, so it provides a worthwhile approach for SNP/indel genotyping of different varieties without prior sequence knowledge, as required by other methods. Results from the wheat genetic diversity assessment confirmed that HRM is a convenient way for a first screening to determine variability groups, prior to resequencing only representative varieties as the basis to develop other SNP platforms. Nevertheless, some HRM limitations have been pointed out. Distefano et al. [88] highlighted that sometimes, the HRM profiles could be similar preventing the differentiation of some of the genotypes. Regardless of this, Wu et al. [98] proposed that this issue can be solved using mixed strategies. Accordingly, our results show that a combined use of different ISBP markers can differentiate all the wheat lines studied.

Twenty ISBP markers for wheat chromosomes 4A and six for chromosome 3B mapped to interesting candidate genes, mainly related to drought and heat stresses and yield components (Tables 2 and 3). They were validated using data from available RNASeq public studies in wheat drought responses [22,26,27], as they showed differences on their expression under different stress conditions.

In chromosome 4A, the ISBP marker *HRM4A_109848074* mapped next (97 bp) to the gene *TraesCS4A01G098300,* which encodes a xyloxyltransferase 1, and participates in carbohydrates metabolism in the development of cellular walls [99]. This process is markedly affected by water stress [100,101] and this can be observed in Figure 5, where this gene decreases its expression under severe stress field conditions. This result is also in agreement with results found by Abebe et al. [102], who analyzed spikes of barley grown under controlled drought conditions, and also found that this gene was down-regulated. The marker *HRM4A_2791416* mapped 814 bp upstream to the gene *TraesCS4A01G003600,* which encodes an alpha/beta-hydrolases superfamily protein with functional adaptability in plants [103]. This gene reduces its expression as drought stress increases under field conditions (Figure 5). Marker *HMR4A_67413676,* mapped 975 bp to the gene *TraesCS4A01G069200,* which encodes an armadillo repeat-only protein. These kind of repeat proteins participate in the coordination of protein interactions during stress and hormonal signalling in plants [104]. In agreement with this, the gene was downregulated under PEG6 drought treatment (Supplementary Materials Table S5). The marker *HMR4A_683608822*, which was located within the drought stress tolerance MQTL32 [71] (Table 5 and Figure 3a), mapped 2685 bp upstream to the gene *TraesCS4A01G410700*, which encodes a ras-related protein RABC2a. The function of this gene has been related to ABA induced stress tolerance in barley [105]. Accordingly to a drought stress response role, this gene was upregulated under PEG6 drought treatment (Supplementary Materials Table S5). The marker *HMR4A_36371442* mapped 2736 bp upstream to the gene *TraesCS4A01G043500* and was downregulated under PEG6 drought treatment (Supplementary Materials Table S5). Contrary to this, this gene increases its expression under severe stress field conditions (Figure 5). This gene encodes a STAS domain containing-protein, which plays a role to membrane attachment of many anion transporters in transport activity and regulation in plants [106]. In fact, it has been demonstrated its crucial role in the activity of sulfate transporter in *Arabidopsis thaliana* [107], providing key amino acids in the sulfate transport activity [108]. The importance of this activity should be noted, since sulfate is an element that has been described as an essential component in the structure of plant enzymes and reserve proteins in grain [109]. Further, marker *HRM4A_716986193*, which was located close to a QTL within MQTL32 [71] (Table 5 and Figure 3a), mapped in proximity (3576 bp upstream) to the gene *TraesCS4A01G671200LC.* This gene encodes a peptidase M20/M25/M40 family protein, which is involved in drought stress responses [110]. In fact, proteolysis under drought conditions allows a reorganization in the plant's metabolism, and also increases plants drought tolerance [20,111,112]. According to this, the results showed increased expression of this gene under different drought stress treatments (Figure 6). This is also in agreement with the results showed by Simova-Stoilova et al. [113], who assessed wheat leaves under severe soil drought and found an increase in peptidase activity. Thus, this drought responsive genes represent an interesting candidate for a known drought and heat stress tolerance MQTL.

Additionally, within the window of +/−20 kb used for the candidate gene analysis in chromosome 4A, there were two interesting genes: *TraesCS4A01G003500* (5141 bp upstream from marker *HRM4A_2791416*) and *TraesCS4A01G047000* (14,885 bp upstream from marker *HRM4A_38654555*). The first gene encodes a thionin like-protein gene, which plays an important role in the growth and development of the plant and its defense against pathogens [114]. It was found differentially expressed under PEG drought treatment, being upregulated under PEG6 drought treatment (Supplementary Materials Table S5). The gene *TraesCS4A01G047000* encodes a formin-like protein, which plays a primary role in the organization of plant's structure [115]. In agreement with our results where this gene was found upregulated under PEG6 drought treatment (Supplementary Materials Table S5), formin-like proteins showed variations in their expression under drought conditions in wheat [115].

Some HRM/ISBP markers mapped to previously described MQTL loci [71] (Table 5 and Figure 3a), which were mainly associated to drought and heat stresses tolerance. Within these markers, *HRM4A_618105078*, which tags the MQTL31 [71] and mapped close (2442 bp upstream) to the gene *TraesCS4A01G497800LC* can be highlighted. This gene encodes a receptor-like protein kinase, which is involved in abiotic stress responses [116], matching the description assigned to the MQTL.

Additionally, within the window of +/−300 kb, four wheat chromosome 3B ISBP markers can be highlighted. The marker *HRM3B_124761338* mapped 6356 bp downstream to the gene *TraesCS3B01G138700*, which encodes a ribonucleoside-diphosphate reductase, an essential enzyme for DNA synthesis [117,118]. This gene increases its expression under severe field stress conditions, and it decreases in response to a PEG drought treatment (Figure 6). Marker *HRM3B_609364064* mapped 31,936 bp downstream to the gene *TraesCS3B01G575000LC*, encoding myosin-1. Plant myosins have a functional role in organelle movement in response to biotic and abiotic stresses [119]. This response is shown in Figure 6, where this gene significantly increments its expression under PEG1 and PEG6 drought treatments. Marker *HRM3B_465802537* mapped to two interesting genes, the gene *TraesCS3B01G290200* (83,072 bp downstream) and the gene *TraesCS3B01G290300* (125,805 bp downstream), which were both found upregulated under PEG6 drought treatment (Supplementary Materials Table S5), and contrary to this, decreased their expression under severe stress field conditions (Figure 6). The gene *TraesCS3B01G290200* encodes a glycosyltransferase, an enzyme which possesses a main role in plant's stress tolerance and defense [120], and in agreement with our results, it has been previously found upregulated in wheat leaf under drought conditions [121]; and the gene *TraesCS3B01G290300* encodes an ABC transporter B family protein, which is significantly involved in organs growth, plant nutrition and development and plant responses to abiotic stresses [122]. In fact, as Rampino et al. [123] highlighted, and in agreement with our results (Supplementary Materials Table S6), the up-regulation of this gene in wheat under heat and drought conditions confirms the implication of this gene family in drought responses. Therefore, this family protein has been related to grain formation in wheat [124], which is consistent with the location of marker *HRM3B_465802537* within MQTL26 [71], mainly related to yield components. Thus, this marker can be useful in wheat breeding, for the marker-assisted selection of this interesting gene and MQTL. Finally, the marker *HRM4A_273339424* mapped 89,674 bp upstream to the gene *TraesCS3B01G221100*, which encodes a protein kinase superfamily protein. This protein's family is involved in plants' responses to abiotic stresses and plants' development [125]. According to this, this gene was found downregulated under PEG6 drought treatment (Supplementary Materials Table S5), and it also shows differences in its expression across different stress conditions (Figure 6). Therefore, these results agree with Wei et al. [126], who highlighted that kinase proteins are involved in various responses with exposure time of drought.

According to our results, the developed HRM-ISBP markers can be used in wheat breeding programs to genotype interesting genomic regions in a cost-effective manner. These markers can useful resources for marker-assisted selection (MAS) focused on abiotic stress responses and yield components, to tag interesting known QTLs and MQTLs related to drought and heat stresses tolerance, and also yield-related traits.

5. Conclusions

In this work, highly polymorphic ISBP markers for wheat chromosome 4A were successfully developed and applied in a genetic variability assessment of a collection of durum and bread wheats, using the high-resolution melting analysis technique. These HRM-ISBP markers represent cost-effective and efficient tools for wheat breeding programs focused on variability assessments. The obtained results provide an interesting framework for wheat genetic studies and varieties selection. These HRM-ISBP markers have also been shown useful for tagging interesting genes associated to drought and heat stresses tolerance, some of which showed differential expression patterns under stress conditions. In addition, some of these markers can be applied in breeding through marker-assisted selection of

QTL and MQTL related to abiotic stresses as drought and heat, and also yield and yield related traits. The resources and results presented here can also facilitate the understanding of important traits in other species with large genomes.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/9/1294/s1; Table S1. List of durum and bread wheat lines used for variability analysis by high resolution melting. [1]: marker set up analysis; [2]: marker's sequence validation by HRM analysis; [3]: HRM pattern types obtained; IFAPA: Instituto Andaluz de Investigación y Formación Agraria, Pesquera, Alimentaria y de la Producción ecológica; INRA: Institut National de la Recherche Agronomique; and WGRC: Wheat Genetics Resource Center; Table S2. Durum wheat lines used for variability analysis by high resolution melting (HRM); Table S3. Candidate genes tagged by the developed HRM-ISBP markers for wheat chromosome 4A. Genes with expression differences are shown in bold and DE genes are indicated with "*". The positive and negative values in the "Dist" column indicate if the corresponding gene is downstream or upstream of the respective marker. Chr: chromosome location. Dist (bp): distance from the gene to the marker in base pairs; Table S4. Candidate genes tagged by the HRM-ISBP markers for wheat chromosome 3B. Genes with expression differences are shown in bold and DE genes are indicated with "*". The positive and negative values in the "Dist" column indicate if the corresponding gene is downstream or upstream of the respective marker. Chr: chromosome location. Dist (bp): distance from the gene to the marker in base pairs; Table S5. Differential expression (DE) analysis significance parameters for DE genes. Significant values (|lg2FC, β|> 1 and *p*-adjust, Q-value < 0.05) are shown in bold; Table S6. Genetic parameters for wheat chromosome 4A ISBP markers used in durum wheat variability assessment. HRM type: high resolution melting pattern type; No. alleles: number of alleles found with the marker; PIC: polymorphism index content; and *: one of the genotypes was described as "null genotype"; Figure S1. Gene expression analysis under different water stress conditions for all candidate genes located within a +/−20 kb window to the wheat chromosome 4A ISBP markers. Genes with differences on their expression are shown in bold and DE genes are indicated with "*". IF: irrigated field conditions; MS: mild stress field conditions; SS: severe stress field conditions; IS: seedling PEG shock control; PEG1: seedling 1 h PEG stress; PEG6: seedling 6 h PEG stress; AD_C: anther stage irrigated shelter phenotype; AD_S: anther stage drought stressed shelter phenotype; T_C: tetrad stage irrigated shelter phenotype; and T_S: tetrad stage drought shelter phenotype; Figure S2. Gene expression analysis under different water stress conditions for all candidate genes located within a +/−300 kb window to the wheat chromosome 3B ISBP markers. Genes with differences on their expression are shown in bold and DE genes are indicated with "*". IF: irrigated field conditions; MS: mild stress field conditions; SS: severe stress field conditions; IS: seedling PEG shock control; PEG1: seedling 1 h PEG stress; PEG6: seedling 6 h PEG stress; AD_C: anther stage irrigated shelter phenotype; AD_S: anther stage drought stressed shelter phenotype; T_C: tetrad stage irrigated shelter phenotype; and T_S: tetrad stage drought shelter phenotype; Figure S3. Phylogenetic UPGMA tree showing the relationships between 76 durum wheat lines genotyped with 7 wheat chromosome 4A ISBP markers. a) wheat lines are colored based on their geographic zone (Supplementary Materials Table S2) (Center: green; North: blue; North-east: light blue; North-west: dark blue; South: red; South-east: orange; South-west: maroon; and East: purple); b) wheat lines are colored for species (*T. turgidum* subsp *durum* appears in green, *T. turgidum* subsp *turgidum* in orange and *T. turgidum* subsp. *dicoccon* in purple). The colored and vertical lines indicate the differentiated clusters (cluster 1—yellow; cluster 2—blue; cluster 3—dark blue; cluster 4—pink; cluster 5—purple; cluster 6—red; cluster 7—green; cluster 8—grey; cluster 9—light blue; and black lines show the wheat lines that have not been included within any cluster); and c) each dot represents a wheat line, the colors are the same in "a)".

Author Contributions: P.H., P.G., and E.P. conceived the experiments. R.M.-G. and L.P. isolated the DNA. E.P. sequenced ISBP amplicons and designed ISBP markers. R.M.-G. performed the PCR and HRM analyses. S.G. performed the bioinformatics analyses. R.M.-G., S.G., E.P., G.D., L.P., P.G., and P.H. analyzed the results. R.M.-G. and P.H. drafted the manuscript. R.M.-G., S.G., E.P., G.D., L.P., P.G., and P.H. have read and agreed to the published version of the manuscript.

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