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CRISTINA CANTALE*,** – ARIANNA LATINI* – PATRIZIA GALEFFI*

Polymorphisms in the A and B genome copies of the *DRF1* gene are able to cluster a collection of durum wheat lines

Summary – Drought tolerance is one of the main components of yield and its stability and improvement is a major challenge to breeders. Transcription factors are considered the best candidate genes for developing functional markers, since they are components of the signal transduction pathways that coordinate the expression of several downstream genes.

In the present study, we report preliminary results concerning the ability of various SNPs in *DRF1* gene variability to cluster 288 lines of durum wheat. These lines represent a subset of the final Durum Wheat Reference Collection (DWRC) panel, organized and managed by Durum Wheat Genomics and Breeding Expert Working Group, in the frame of the activities of the International Wheat Initiative. The lines were selected among elite and landraces and genotyped by several KASP assays designed using *DRF1* gene polymorphisms. The KASP (Kompetitive Allele Specific PCR) genotyping platform was considered as the most adapt system due to the few SNPs and the large number of genotypes involved.

24 SNPs were identified by comparing several *TdDRF1* gene copies from a large panel of durum varieties and were used to design an original set of KASP assays.

Preliminary results concerning the ability of some SNPs to cluster genotypes are reported.

Keywords: genotyping, wheat, SNP, KASP assay.

Introduction

Climate changes represent an additional challenge for agriculture that is already committed to feed the increasing world population. Plant growth and productivity are greatly affected by abiotic stresses as drought [1]. Plants deploy complex mechanisms to cope stresses: there are genes that directly protect cells by producing

* ENEA CR-Casaccia, Via Anguillarese, 301 - 00123 Rome, Italy.
** Corresponding author: E.mail: cristina.cantale@enea.it

important metabolic proteins and transcription factors and *cis*-acting elements involved in transduction networks, from the signal perception to the target gene expression. Improved adaptation to drought may depend upon efficient signal perception and adequate responses at transcriptional level [2, 3].

The dehydration responsive element binding (*DREB*) family of transcription factors is involved in cold, salt and drought stress responses [4]. These proteins interact with the DRE motif in the promoter regions of many stress inducible genes and belong to the larger AP2/ERF family, as the DNA recognition and binding is mediated by the Apetala2 (AP2) domain.

A *DREB*-related gene, namely *TdDRF1* (*Triticum durum Dehydration Responsive Factor* 1), was isolated in durum wheat and its expression was related to the response to water deficit. It is homologous to *DREB2* gene and its structure consists of four exons and three introns that produce three transcripts by an alternative splicing mechanism [5]. This gene structure is present also in the ancestors, donors of A and B genomes, in *A. tauschii*, donor of D genome, and in other members of *Triticae* tribe, as bread wheat and barley.

Transcription factors are considered optimal candidate genes to target in the development of functional markers (FMs) [6] and sequence variability in these regulatory genes is expected to have a relevant influence on the variation for complex traits [7, 8].

Recently, we were able to sequence the two copies of the gene in A and B genomes in two unrelated cultivars [9]. In the present work, we report the punctual analyses of the above mentioned four sequences that revealed several distinctive features characterizing each genome copy. Many polymorphisms were identified: most represented simply sequence variants between homeologous genomes, that are intravarietal SNPs that cannot represent markers, but some singletons, variants between homologous genomes of the two cultivars (intervarietal), could be useful for developing markers. Furthermore, analysing more sequences from other cultivars, new additional varietal SNPs were identified. The final set of interesting SNPs needed to be more investigated using a larger germplasm. A subset of 288 accessions from the final Durum Wheat Reference Collection (DWRC) panel (made available by International Wheat Initiative, Expert Working Group Durum Wheat Genomics and Breeding – EWG-DWGB) was selected and used to investigate the distribution of the *TdDRF1* alleles.

Among the different SNP genotyping platforms, the kompetitive allele-specific PCR (KASP) platform resulted the most suitable for the present study. Specific KASP primers were designed from sequences of the two allelic variants and used to perform the KASP assays. Here we report the preliminary results.

Materials and methods

Germplasm. 288 DNA samples belonging to the DWRC were kindly provided by EWG-DWGB of International Wheat Initiative. This subset was selected from

the whole 960 final panel DNA collection and included all elite lines and few landraces.

Genotyping. The KASP (Kompetitive Allele Specific PCR) technology of LGC Limited [10, 11] was chosen as genotyping platform. It is based on allele-specific oligo extension and fluorescence resonance energy transfer (FRET) for signal generation. The fluorescent reporting system includes four single-labelled oligonucleotides that hybridize to one another in free solution to form a fluorescent quenched pair, which upon introduction of complementary sequence, generates a measurable signal [12].

A large number of *DRF1* gene sequences from different durum wheat varieties were available in our laboratory and were aligned by Clustal Omega in order to identify interesting polymorphisms.

Twenty-four polymorphisms were used to design KASP assays and KASP primers were developed following standard KASP guidelines. The allele-specific primers were designed carrying the standard FAM (5′ GAAGGTGACCAA GTTCATGCT 3′) and HEX (5′ GAAGGTCGGAGTCAACGGATT 3′) tails and with the targeted SNP at the 3 end. The allele-specific and a common primer were designed able to amplify a fragment about 120 bp.

The PCR mix included: 2.5 μl DNA (about 10 ng/μl), 2xMastermix 2.5 μl and primer mix 0.07 μl. PCR cycling was performed using the following protocol: hot start at 94 °C for 15 min, followed by 10 touchdown cycles (94 °C for 20 s; touchdown at 61 °C initially and decreasing by -0.6 °C per cycle to achieve a final temperature of 55° C), followed by 26 additional cycles of amplification (94 °C for 20 s, 55 °C for 60 s). In some cases, according to primer nucleotide composition, touchdown temperatures was changed to 68-62 °C and, accordingly, the final temperature of amplification stage was 62° C.

Results

In Table 1, the list of polymorphisms identified in expressed regions of the gene is shown. All of them mapped Exon 4 except one, namely Co2, mapping Exon 2. The analysis excluded intravarietal SNPs, corresponding to sequence variants between A and B genomes. Only 7 out 24 SNPs represented synonymous substitutions. It is worth of noting that 3 out 17 replacement substitutions lay in the region codifying for the highly conserved AP2 domain, responsible for DNA recognition and further 2 replacements involved nucleotides codifying for amino-acids flanking the same domain.

In Table 2 the alleles identified in the analysed genotypes are listed. 3 out of 24 assays, namely V5, V6 and C5, showed an unspecific amplification and alleles could not be assigned. In 19 assays, just one of the two alleles was present, while in 2 assays, namely K2 and C6, both alleles were identified.

ASSAY ID	AlleleX	AlleleY	POLYMORPHISM EFFECT
Co2	G	A	replacement
C1	A	G	replacement
C2	G	A	replacement
V3	A	C	replacement
C3	A	G	replacement
V4	**A**	**G**	**replacement**
V5	**A**	**C**	**replacement**
M1	**A**	**G**	**replacement**
V6	G	C	replacement
M2	G	A	synonymous
C4	C	T	replacement
C5	GGAGT	TACTC	replacement
V7	G	A	replacement
V8	C	A	replacement
K1	T	C	replacement
V9	C	T	synonymous
K2	G	A	replacement
C6	A	C	replacement
C7	A	G	synonymous
C8	T	C	synonymous
V10	T	A	synonymous
M3	G	A	synonymous
M4	C	T	synonymous
Co1	AAAA	GAAG	replacement

Table 1. Polymorphisms identified in the expressed regions of the *TdDRF1* gene, listed accordingly to their position along the gene, the first one being in Exon 2, the other ones in Exon 4. The effect of the polymorphism on the amino-acid translation is also shown. In bold the polymorphisms included in the AP2 domain codifying region are highlighted.

ASSAY ID	AlleleX	AlleleY
Co2		A
C1		G
C2		A
V3		C
C3		G
V4		G
V5	/	/
M1		G
V6	/	/
M2		A
C4		T
C5	/	/
V7		A
V8		A
K1		C
V9		T
K2	G	A
C6	A	C
C7		G
C8		C
V10		A
M3		A
M4		T
Co1		GAAG

Table 2. Alleles identified in the 288 analysed genotypes. / stands for no allele assignment.

Discussion

The investigation of genetic diversity of the *TdDRF1* gene was carried out analysing the collection of sequences available in our laboratory. The isolation and sequencing of A and B genome copies of the gene [9] allowed to recognize homeologous (intravarietal) and homologous (intervarietal) SNPs, at the base of designing new markers. We selected 24 intervarietal SNPs as interesting polymorphisms to be investigated in view of a possible association with phenotypic data as a first step for developing new markers. Looking to the sequence of resulting proteins, most of selected 24 SNPs produced replacements in the amino-acid sequences, only 7 being silent. The availability of a large collection of durum wheat elite lines allowed us to investigate the distribution of these selected alleles in a larger germplasm. The KASP assay was chosen as the most reliable genotyping platform, because few SNPs had to be analysed in a quite large number of genotypes.

The most part of assays, namely 19, was not able to distinguish the genotypes, due to the presence of just one allele. It is worth of noting that two KASP assays were able to cluster the genotypes into two groups, meaning that both alleles were present: a validation is necessary to associate a specific allele with a trait of agronomic/industrial interest, in view of developing a marker.

The validation is foreseen in the frame of activities of EWG group that provided the germplasm collection and is currently engaged in its phenotyping.

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