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HRM technology for the identification and characterization of INDEL and SNP mutations in genes involved in drought and salt tolerance of durum wheat

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Abstract

WRKY transcription factors are one of the largest families of transcriptional regulators and form an integral part of signalling webs which modulate many plant processes, such as abiotic stress tolerance. In the present paper, an innovative method has been applied to identify novel WRKY-1 alleles involved in the responses to salt and drought stresses in *Triticum durum*. This technique involves scanning for sequencing variations in cDNA-derived PCR amplicons, using high-resolution melting (HRM) followed by direct Sanger sequencing of only those amplicons which were predicted to carry nucleotide changes. HRM represents a novel advance in detection of single-nucleotide polymorphisms (SNPs) by measuring temperature-induced strand separation of short PCR amplicons. The use of this approach is still limited in the field of plant biology. Here, HRM analysis has been applied to the discovery and genotyping of durum wheat SNPs. Specific primers have been designed, starting at multi-alignment of WRKY-1-conserved portions. The PCR amplicons, containing single SNPs, produce distinctive HRM profiles, and by sequencing the PCR products identified, SNPs have been characterized and validated. The results showed that all the revealed SNPs are located on salt-tolerant varieties, confirming their value in breeding activities.

Keywords: durum wheat; high-resolution melting; salt tolerance; single-nucleotide polymorphisms

Introduction

Environmental stresses, such as salinity, are the cause of great losses in crop yields every year all over the globe (Boyer, 1982). Therefore, salt-responsive or salt-tolerance mechanisms have been intensively studied from biological and genetic perspectives. Signal transduction pathways regulate reactive oxygen species, damage repair and ion homeostasis, while maintaining a low Na^+/K^+ ratio is important for plant survival under salt stress (Kader *et al.*, 2006). Molecules that have a function in the adaptation to environmental stresses can be divided into two groups: the effector molecules and the regulatory molecules (Hasegawa and Bressan, 2000). In particular, WRKY factors are one of the largest families of transcriptional regulators in plants and form integral parts of signalling webs that modulate many plant processes, including the responses to abiotic stresses, such as drought, cold and salt stresses. The WRKY family is among the ten largest families of transcription factors in higher plants and is found throughout the green lineage (Rushton *et al.*, 1996). Recent studies have confirmed that WRKY proteins often act as repressors as well as activators and that the members of the family play a role in both repression and depression of important plant processes (Ishiguro and Nakamura, 1994). Furthermore, it may be that a single WRKY transcription factor is involved in regulating several seemingly disparate

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processes. The defining feature of WRKY transcription factors is their DNA-binding domain, called WRKY after the most unvaried WRKY amino-acid sequence at the N terminus (Rushton *et al.*, 1996). The WRKY domain consists of a four-stranded β-sheet, with the zinc coordinating Cys/His residues forming a zinc-binding pocket, enabling access to the major DNA groove and contacting with the DNA. High-resolution melting (HRM) analysis has been developed to detect single-nucleotide polymorphisms (SNPs) in small PCR amplicons because it is an easy and low-cost method. Up to now, it has been principally used for scanning mutations of large multiple exon genes to identify disease-related mutations in humans (Kennerson *et al.*, 2007). In the area of plant biology, the use of this technique is still limited and has been applied exclusively for constructing linkage map (Chagné *et al.*, 2007; Croxford *et al.*, 2008). This technique measures temperature-induced strand separation, and is therefore able to detect variations as small as one base difference between samples. The evolution of the HRM technique from traditional melting curve analysis has been implemented through the invention of new-generation intercalating dyes which can saturate-bind to the double-stranded DNA without PCR inhibition. In this study, we have searched for the presence of SNPs in durum wheat varieties with different degrees of tolerance to salt stress. Conserved portions of WRKY-1 gene were selected through a multi-alignment of homologue sequences of the gene in wild relatives of the species; specific primers have been designed in order to obtain amplicons not longer than 100 bp analysed by HMR technique.

Materials and methods

Four durum wheat varieties, Cham I (moderately salt tolerant), Jennah Khetifa (salt tolerant), Belikh 2 (moderately salt tolerant) and Trinakria (salt susceptible) were used for SNPs discovery and validation in the WRKY-1 gene. Plants were grown with a nutritive solution for 7 d, after which NaCl was added at different concentrations: 0 M (as a negative control), 0.75 and 1.5 M. RNA were then extracted from leaf and root material using TRIzol® reagent (Invitrogen) following the manufacturer's protocol. The cDNA was synthesized using oligo(dT)-primers and the SuperScript™ III reverse transcriptase (Invitrogen), according to the manufacturer's instructions; the reactions were subsequently treated with RNaseH (Invitrogen). The cDNA obtained was used in HRM amplifications. Primers employed in HRM analysis were designed utilizing the software Primer3, starting from a multi-alignment of the sequences, which characterize the conserved domain of the WRKY-1 gene close to WRKY site, in 14 wild relative species. Two primer pairs were designed to cover a conserved domain of 250 bp. PCR amplifications were performed

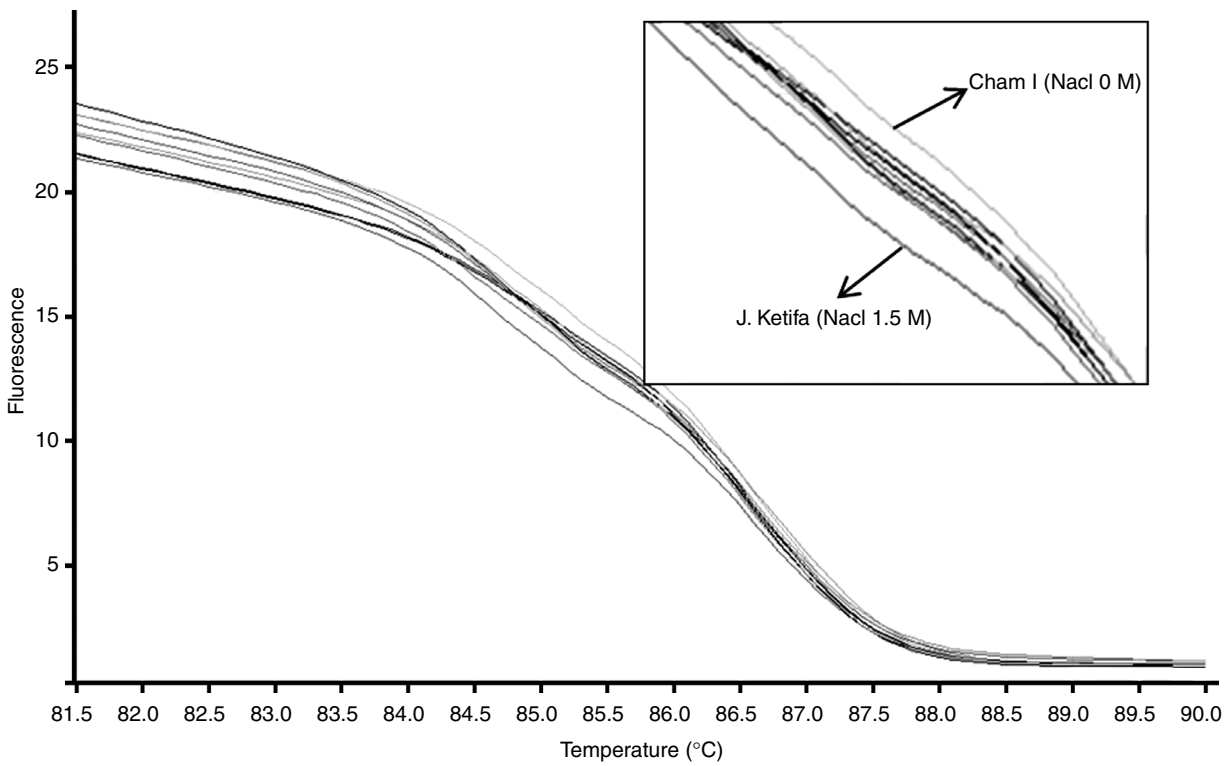

Fig. 1. WRKY-1 melting curve profiles.

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on a Rotor-Gene 6000 realtime PCR Thermocycler (Corbett Research, Australia). For data quality control, PCR amplification was analysed through the assessment of the threshold cycle (Ct) value, endpoint fluorescence level and the amplification efficiency. The melting data were normalized by adjusting the beginning and end fluorescence signals to the same level. HRM curve analysis was performed using the HRM analysis module. Different plots of the melting data were visualized by selecting a genotype for comparison and negative first-derivative melting curves were produced from the fluorescence *versus* temperature plots. Representative genotypes were chosen for sequencing, using an ABI 3130xl sequencing platform. Sequences obtained were blasted with the corresponding sequences present in genes databases through BLAST2 Sequences program and multi-aligned to identify mutations.

Results and discussions

A total of 14 sequences relative to a conserved WRKY-1 domain were multi-aligned for primer design. There were 36 amplicons corresponding to the cDNA extracted from leaves and roots of the different varieties treated with the different salt concentrations. HRM analysis revealed that eight amplicons showed polymorphic melting curves when assayed against non-treated samples (negative controls), while the remaining 28 were monomorphic (Fig. 1). Nine amplicons, in two replicates, showing both mono- and polymorphic melting curves, and analysed on normalised melting curves and through different plots, were chosen, purified and sequenced. Among these, two SNPs were found in salt-tolerant lines Cham I and J. Ketifa both in leaves and roots. The first SNPs present in an A/T transversion were located close (only one amino acid distant) to the WRKY domain. The A/T transversions are commonly considered to be the SNPs variations most difficult to resolve by melting analysis. Despite this, the current study showed the amplicon with the A/T transversion was distinctly differentiated (Fig. 1). Moreover, this SNP was present in the salt-tolerant line, Cham I, even in the NaCl-untreated condition. A second SNP, consisting of a G/C transversion, was located on the other resistant line, J. Ketifa, treated with the maximum concentration (1.5 M) of NaCl. Also, this SNP was located close (only two amino acid distant, adjacent to the first SNP) to the WRKY domain. This SNP also showed a distinctive melting curve profile (Fig. 1). Both of the identified SNPs created two corresponding amino acid substitutions, Q/L for the first SNP and K/N for the second (Q = Gln = Glutamine, L = Leu = Leucine,

Fig. 2. Amino acid and nucleotide sequences of WRKY-1 with WRKY domain (grey box) and the two different SNPs found for Cham I (round box) and J. Ketifa (squarely box).

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K = Lys = Lysine, N = Asn = Asparagine), both close to the WRKY domain (Fig. 2). The presence of these two altered amino acids so close to WRKY may cause a functional modification in the biochemistry of the WRKY-1 promoter (considering that this protein site is involved in DNA binding) that could explain and contribute to the resistance of these two durum wheat lines to salt stress. The presence of the first SNP in the Cham I line, in the untreated condition, may be explained by WRKY-1 being part of one of the largest families of transcription factors. These factors are involved in regulating several seemingly disparate processes where some proteins act as repressors as well activators (Rushton *et al.*, 2010). Furthermore, understanding the different mechanisms of WRKY transcription factors is straightforward, as reported by Ishiguro and Nakamura (1994) and Rushton *et al.* (2010). Nevertheless, the localization of both SNPs in both the resistant lines confirms their value for future breeding activities. In addition, this work confirms that HRM represents a feasible means for SNP detection and genotyping, being a simple, accurate, high-throughput and low-cost method.

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