Real-Time PCR, a tool for the analysis and quantitation of WIS2-1A retrotransposon in hulled wheat

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ABSTRACT

The Real-time PCR technique was adopted to assess the genetic variability present in five accessions of einkorn (*T. monococcum*, 2n = 2x = 14), emmer (*T. turgidum*, 2n = 4x = 28) and spelt (*T. spelta*, 2n = 6x = 42). A simple Real-time PCR assay, based on SYBRGreen I dye, was employed to detect the copy number of one of the most important retrotransposons present in *Triticum* genomes, WIS2-1A. It is the first retrotransposon found in wheat and was primarily observed as an insertion into a High-Molecular-Weight (HMW) storage protein gene in *T. aestivum*. It represents an ancient DNA element that probably was already present in the common diploid ancestor of the *Triticeae* tribe. In the present work, it has been developed and optimized using a Real-time PCR assay which has permitted the detection of the presence and the number of retrotransposons. Significant differences were observed in the WIS2-1A copy number both among species and among accessions within species. Furthermore, as expected, the lowest copy number was observed for *T. monococcum* which represents the diploid level present among hulled wheat. On the other hand, a similar number of copies has been observed in *T. dicoccum* (tetraploid) and in *T. spelta* (hexaploid). In previous studies in barley, a strong correlation between the retrotransposons copy number and genome size was observed; recently in wheat it has been demonstrated that the wheat genome A has a higher transposable element content than genomes B and D. Therefore, this work confirms previous results where it has been observed that the A ancestral genome may have under-gone differential genome expansion caused by Class I elements prior to speciation of the tetraploid wheat ancestor; hence the amount of retrotransposon is not linearly linked to the ploidy level of the wheat species.

INTRODUCTION

Plant retrotransposons are grouped into two distinct classes. Class I, commonly referred to as retroelements or retrotransposons, are characterized by the presence of a long terminal repeat (LTR) and movement via RNA intermediate. Class II elements, or LINE (Long Interspersed Nuclear Elements)-like retrotransposons, transpose by a cut-and-paste mechanism with no RNA intermediate; they have no LTR, but are flanked by terminal inverted repeats. Although transposon insertion can have deleterious effects on the host genome, transposons are considered important for adaptative evolution, and can be instrumental in the acquisition of novel traits[1]. Retrotransposons are particularly important in the *Triticum* species, since about 80% of the wheat genome consists of repetitive elements[2]. WIS 2-1A was the first retrotransposon to be identified in wheat[3], in the form of an 8kbp insertion within a high-molecular-weight glutenin subunit gene[4]. It is common in hortologous sequences, and shows a high level of intergenomic but a low level of intragenomic variability[5]. Its insertion site is flanked by a 5bp duplication, with an LTR of 500bp, and its termini contain an almost identical, but inverted 6bp sequence, beginning with TG and ending with CA. WIS 2-1A belongs to the *Ty1-copia* family, but has lost its capacity to transpose autonomously, as a result of the accumulation of mutations[6]. In addition, it appears to have no specific insertion target, or at least, its stringency is too low to prevent the insertion into other parts of the genome. WIS 2-1A is only rarely trans-activated *in vivo*, and is clearly an ancient DNA element, probably already present in the diploid progenitor of the *Triticeae* tribe[5]. The copy number of WIS 2-1A has been estimated to be about 200 per haploid genome in bread wheat[5]. The detection of variation in retrotransposon copy number to date has been achieved by assessing signal intensity either at the endpoint of a PCR reaction or in restriction digests, or by PCR-ELISA. The latter represents the standard tool for the quantification and analysis of gene expression[7]. The development of real time PCR has made possible the direct monitoring of the amplification reaction during its progress. We present here the real time PCR protocol optimization and its utilization for quantifying retrotransposons copy number in the three ploidy levels of hulled wheat.

MATERIALS AND METHODS

Genomic DNA was isolated, following the Dvorak *et al.*[8] procedure, from leaves of five accessions of each of the three hulled wheat species, einkorn (*T. monococcum*, 2n = 2x = 14), emmer (*T. turgidum*, 2n = 4x = 28) and spelt (*T. spelta*, 2n = 6x = 42) (Table 1). Both quality and concentration of the DNA were assessed by agarose gel electrophoresis and spectrophotometry. Functional primer pairs were generated to amplify fragments of WIS 2-1A [5](5'-AAGAAAGGTTGT ATGTGATA-3', 5'-GTCAACAACATATACTCATC-3'. Reactions were made up to a final volume of 10µl, containing 10ng template DNA, 0.6µM of each primer, 100µM dNTP, 5mM $MgCl_2$ and 0.5U *Taq* DNA polymerase. The amplification regime consisted of an initial denaturation step of 95°C/2min, followed by 45 cycles of 94°C/1min, 52°C/2min and 72°C/2min, ending with an extension step of 72°C/10min. PCR products were separated by both agarose and polyacrylamide gel electrophoresis,

and visualized following ethidium bromide staining and fluorescence labeling, respectively.

Table 1. Genotypes of hulled wheat used in this study and WIS 2-1A copy number, expressed per ng of genomic DNA in the hulled wheat accessions analyzed.

Accession	Species	Genome	Copy Number /ng gDNA
ID 189	*T. monococcum*	A	1
ID 59	*T. monococcum*	A	2
ID 44	*T. monococcum*	A	3
ID 41	*T. monococcum*	A	6
ID 63	*T. monococcum*	A	10
Leonessa	*T. dicoccum*	AB	11
Molise	*T. dicoccum*	AB	15
Umbria	*T. dicoccum*	AB	17
Potenza	*T. dicoccum*	AB	19
Garfagnana	*T. dicoccum*	AB	27
Sp. Altgold	*T. spelta*	ABD	19
Sp. Forenza	*T. spelta*	ABD	22
Sp. Frunken	*T. spelta*	ABD	23
Sp. Balmeg	*T. spelta*	ABD	26
Sp. Ebner	*T. spelta*	ABD	27

RESULTS

The electrophoretic profiles of the WIS 2-1A amplicon were multi-banded (Figure 1), with a clear differentiation between einkorn and emmer plus spelta samples. The intraspecific level of polymorphism was low and comprised largely of quantitative variation in band intensity.

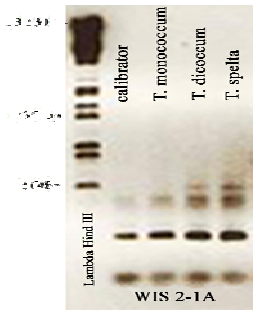

Figure 1. Conventional PCR profiles generated with primers targeting WIS 2-1A in hulled wheat.

The conventional PCR reaction conditions were not suited for the LightCycler 2.1 fluorimeter (Roche), and therefore needed to be redefined. For an accurate quantification of samples for which the copy number is unknown it was important to start with a concentration of template DNA equal to the concentration of the calibrator (5 ng of genomic DNA). Successful amplification was achieved in 20µl reactions, containing 5ng template DNA, 1µM of each primer, 5mM $MgCl_2$ and 10µl of master mix (LightCycler DNA Master SYBR Green I, Roche Diagnostics; Quantitect SYBR Green PCR Kit, QIAGEN). A pre-incubation of 95°C/15min was followed by 45 cycles of 94°C/15s, 48°C/20s and 72°C/30s, and fluorescence was detected at 88°C at the end of each cycle. A melting point analysis was performed in the range 65°C - 95°C. A preliminary screen showed that the einkorn accession ID 189 had the lowest retrotransposon copy number; it was therefore used as calibrator. The standard curve was constructed from a serial 10-fold dilution of 0.005 to 50ng of this DNA. The relative quantification of retrotransposon copy number was obtained using a 200bp fragment of the single copy Glu-A1y gene as a reference amplicon (primer sequences kindly provided by Prof. R. D'Ovidio). This choice was due to the fact that Glu-A1y is wheat-specific with a single copy (200 bp) per haploid genome. The model real-time PCR amplification curve of fluorescence intensity against cycle number was sigmoidal (Figure 2), with the amplification product from the early cycles remaining below the limit of detection. However, assay progress was monitored from the onset of the log-linear phase. At the "threshold cycle" (C_T), fluorescence became detectable, and since the cycle at which this occurred was dependent upon the quantity of template, C_T was used to estimate template quantity during the quantitative real-time PCR progress. Copy number was then determined by comparing the amplification curve, derived from each test sample, with the standard curve. Product quantification was based on measured C_T values, and the resulting data were analyzed with RelQuant (Relative Quantification) software (version 2.1, Roche Diagnostics). One-way Analysis (SPSS version 14 software) was performed to test differences in copy number between ploidy levels.

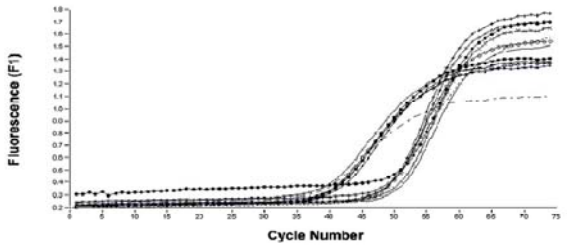

Figure 2. Examples of amplification curves obtained from targeted retrotransposon and reference gene primers. The plot shows the relationship between fluorescence intensity (logarithmic scale) and cycle number.

Two main factors - $MgCl_2$ concentration and annealing temperature - influenced PCR yield and specificity. Decreasing the annealing temperature to 48°C and deleting the final elongation step produced a great reduction in the experimental noise. In addition, the primer concentration had to be raised to increase yield. The optimized cycling protocol resulted in satisfactory amplification of the expected products (example shown in Figure 4). Signal could be detected from 5pg or more of genomic DNA. The crossing points are shown in Table 2. The correlation coefficient of the curve was -0.98 for WIS 2-1A products. Melting curves were calculated for each PCR run. A sudden decrease in

fluorescence occurred when the duplex amplicon melted. Thus the melting curve revealed a specific pattern and an accurate estimate of Tm for each target. Based on these results, the real-time PCR conditions were optimized to minimize non-specific amplification and maximize PCR efficiency (slope ranging from -2.9 to -4.5). Thus, the melting curve in Figure 4 shows how non-specific amplicons were removed when the Tm was raised to 78.5°C. The melting curve characteristics were also used to evaluate the specificity of the PCR product. The copy number of WIS 2-1A retrotransposons in einkorn varied from 2 - 10 per ng of template, from 11 - 28 in emmer and from 19 - 27 in spelt (Table 1).

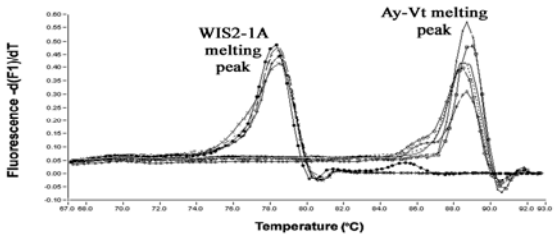

Figure 2. Examples of melting curve analysis of the WIS2-1A and reference gene primers. Melting curves were generated after 45 amplification cycles at an annealing temperature of 50°C, and the analysis was performed over the range 65°C - 95°C.

DISCUSSION

Polyploidization, invasion of transposable elements and expansion of repetitive DNA all contributed to the size of the *Triticeae* genomes[9]. The replicative spread of retrotransposons promoted insertional polymorphism, increased retrotransposon copy number, and contributed to genome diversification[7]. Differences in the conventional amplification profiles displayed by einkorn and emmer indicated a high level of insertional polymorphism associated with the WIS 2-1A retrotransposon. The complexity of the profile is the end result of polymorphism in insertion and excision points, as well as point mutations in the LTRs. In the present work we have developed a real-time PCR approach to derive the copy number of WIS 2-1A elements. Real-time PCR assay has advantages over conventional methods based on agarose gel electrophoresis: it does not includes a number of laborious post-PCR handling steps, its specificity is directly confirmed by melting curve profiling, and it has a great sensitivity. Significant differences were observed in the WIS 2-1A copy number both intra- and inter-specifically. WIS 2-1A profiles have been reported to be highly species specific and to show almost no intraspecific variation[5]. However we were able to observe a low level of intraspecific polymorphism, both on agarose and polyacrylamide gels. Banding patterns were qualitatively very similar, but differences in band intensity were detectable, indicating the presence of polymorphism for copy number. As expected, einkorn, which has the lowest ploidy level, had the lowest WIS 2-1A copy number. Emmer and spelt wheat had similar copy numbers, in line with the results reported by Moore *et al*.[4] who demonstrated in species such as *T. aestivum* (A, B and D genomes), *Ae. longissima* (S genome), *Ae. squarrosa* (the proposed donor of D genome), and *T. monococcum*, that the B genome has a higher number of WIS 2-1A retroelements than the A and D genomes. On the basis of the known 1C DNA content of einkorn, emmer and spelt[10] (respectively, 6.2pg, 12.0pg and 15.7pg), it was possible to indicate that the WIS 2-1A copy number per unit of DNA content was similar in the three species, although in einkorn (161 - 1612) it was somewhat lower than in the other two species (emmer 916 – 2250; spelta 1210 - 1720). Nuclear DNA content is double in the cell cycle S-phase; thus, C-values differ by 1:2 depending upon cell phase. Moreover, species have different amounts of 'C' due to their polyploidy level; hence, it is 2 or 4 C for diploid, 4 or 8 C for tetraploid and 6 or 12 C for hexaploid. These values differ markedly from the estimate of 200 copies per haploid genome reported by Monte *et al*.[5] utilizing *in-situ* hybridization technique.

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