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in

Molina-Cano J.L. (ed.), Christou P. (ed.), Graner A. (ed.), Hammer K. (ed.), Jouve N. (ed.), Keller B. (ed.), Lasa J.M. (ed.), Powell W. (ed.), Royo C. (ed.), Shewry P. (ed.), Stanca A.M. (ed.).
Cereal science and technology for feeding ten billion people: genomics era and beyond

Zaragoza : CIHEAM / IRTA
Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 81

2008
pages 441-444

Analysis of the expression of homoeologous genes in polyploid cereals using fluorescence cDNA-SSCP

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SUMMARY – Most cereals have genomes with redundant genes arising from genome doubling or polyploidy. Differences in the expression levels of duplicate genes have long been a subject of great interest since new genes can arise from redundant duplicates. Such analysis requires a technique that can discriminate between the expressions of two (or more) almost identical genes (homoeologues). In previous works we isolated and characterized the sequences of the gene *Mre11* in the genomes A, B and D of *Triticum aestivum* L., involved in the homologous recombination mechanism. Herein we report the results of application of an improved method based on the cDNA-SSCP technique reported by Cronn and Adams (2003), using fluorescence labelling and automatic sequencing. The results obtained in both tetraploid and hexaploid species indicated a lower expression of *Mre11* gene from the B genome respect to genes from genomes A and D.

Introduction

Most higher eukaryotes have genomes with redundant genes arising from genome doubling or polyploidy (Adams *et al.*, 2003). Differences in the expression levels of duplicate genes have long been a subject of great interest since new genes can arise from redundant duplicates (Wen-Hsiung, 2005). The accurate assessment of the expression of each member of a homoeologous gene pair is therefore of great importance.

Such analysis requires a technique that can discriminate between expression of two or more almost identical genes (homoeologues). Northern blot analysis, the most commonly used technique for gene expression analysis is inadequate. Recently, Cronn and Adams (2003) developed a PCR-SSCP technique for the characterization and quantification of the relative expression of homoeologous genes in polyploid cotton. Herein we report the modification and improvement of this technique using fluorescence labelling and an automatic sequencer to analyse expression of the *Mre11* gene involved in homologous recombination in polyploid wheat species.

Materials and methods

Plant material

The diploid species *Triticum monococcum* (genome A) and *Aegilops tauschii* (genome D), the tetraploid species *Triticum turgidum* cultivar 'Vitron' (genomes A and B) and the hexaploid species *Triticum aestivum* cultivar 'Chinese spring' (genomes A, B and D) were used in the study.

RNA isolation, cDNA synthesis and SSCP analysis

For RNA isolation, immature spikelets were collected at meiosis when the tip of the spike was level with the ligule of the penultimate leaf, which indicated that the microspores were in the mid- to late uninucleate stage (confirmed by microscopy). Total RNA was purified using Tripure reagent (Roche) following the protocol provided by the supplier. To eliminate the contaminating DNA, RNA was purified using the Turbo DNA-free Kit (Ambion). First strand cDNA synthesis was carried out from 5 µl of DNA-free RNA (~2 µg of input RNA) using Transcriptor Reverse Transcriptase and Oligo $(dT)_{15}$ primer (Roche) following the protocol provided by the supplier. To obtain the cDNA *Mre11* sequences, 2µl of

first strand cDNA were used in a PCR reaction containing the Mre11-L and Mre11-R primers (5'-TGCCATCTGGGCTACATGGA-3' and 5' GAGAATCATCTCCTCTTGAC 3' respectively).

Fluorescence SSCP technique was developed based on the radioactive technique described by Cronn and Adams (2003). Modifications of the original technique consisted mainly in the amplification with fluorescence labelled primers and the use of an automatic sequencer. First, amplification from *Mre11* cDNA was carried out using primers SSCP-L: (5' AGAGGTAAATCTGTAGCAGC 3') and SSCP-R2: (5' GGATCTGAATTTTCAACAACTTC 3') without labelling. PCR was performed in a reaction volume of 30 µl including 2U of Taq polymerase and 1x buffer (Sigma), 100 µM dNTPs, 0.3 µM of each primer and 1 µl of cDNA. The PCR cycling conditions consisted of an initial incubation for 2 min at 95℃, followed by 35 cycles of 30 s at 94℃, 30 s at 57℃ and 30 s at 72℃, and a final extension step at 72℃ for 5 min. The amplified products were loaded onto agarose gels and purified using the High Pure PCR Product Purification Kit (Roche). After purification, the products were labelled by PCR using the same primers as above, but fluorescently labelled with 6 FAM. This reaction was performed using 0.5 µM of a single labelled primer, in a volume of 10 µl containing 0.6 U and 1x Taq Buffer (Sigma), 175 µM dNTPs, and 5-8 ng of purified DNA fragment. PCR cycle was as above, after which the reaction volumes were diluted (1:10) and 5 µl of each were mixed with 20 µl of formamide and 0.5 µl of standard Rox500 (Applied Biosystems). Fluorescence analysis was performed in an ABI 3130 Avant Genetic Analyzer using a 50 cm capillary array and a performance optimised polymer (POP-7) (Applied Biosystems). The default settings were used except for temperature (18℃) and time (3500 s). The analysis was performed in triplicate and cDNA was synthesised for each PCR. The proportion of each transcript was related to the fluorescence area of each peak.

Results and discussion

The SSCP technique was used to analyse the relative expression of homoeologues genes in each wheat polyploid species. To evaluate the accuracy of the modified SSCP fluorescence technique, known amounts of the transcripts of two *Mre11* homoeologous genes belonging to the A and B genomes of *T. turgidum* were analysed in the same way as reported by Cronn and Adams (2003) where the level of expression observed with this technique was in accordance with the amount of transcripts used. In our case, cDNA clones of the *Mre11* genes were used to amplify a 237 bp fragment using the unlabelled SSCP-L and SSCP-R2 primers.

The A and B genome fragments are identical in size but differ by 9 substitutions, yielding a pairwise nucleotide identity of 96.2%. Mixtures of purified A and B PCR products were then made up in the following ratios (8 µl volume): 5 ng A + 0.05 ng B (100:1), 5 ng A + 0.25 ng B (20:1), 5 ng A + 0.71 ng B (7:1), 5 ng A + 1.66 ng B (3:1), 5 ng A + 5 ng B (1:1), 1.66 ng A + 5 ng B (1:3), 0.71 ng A + 5 ng B (1:7), 0.25 ng A + 5 ng B (1:20), and 0.05 ng A + 5 ng B (1:100). In addition, 5 ng of fragments A and B were used separately as controls. For each mixture, three independent labelling reactions were performed.

Gene expression is related to the area of the fluorescence peak. Expression values were obtained by calculating the average of three replicates. Corrections for background were sometimes required for the 1:20 and 1:100 dilutions. Figure 1 shows the results of one of the three replicates for all the mixtures performed. The technique clearly separates two peaks in each mixture, representing the transcripts of the genes *Mre11* of both genomes. The technique showed good reproducibility, as shown by the very small standard deviations observed (Table 1). The comparison of these results with those of Cronn and Adams (2003) shows the proposed fluorescence technique to be more reproducible and more accurate.

In order to validate the method, genomic DNA of tetraploid *Triticum turgidum* was analysed. Identical level of fluorescence would be expected for both genes. Values of 44.6% and 55.4%, close to those expected for the *Mre11* genes of the A and B genomes, respectively, were obtained validating the technique.

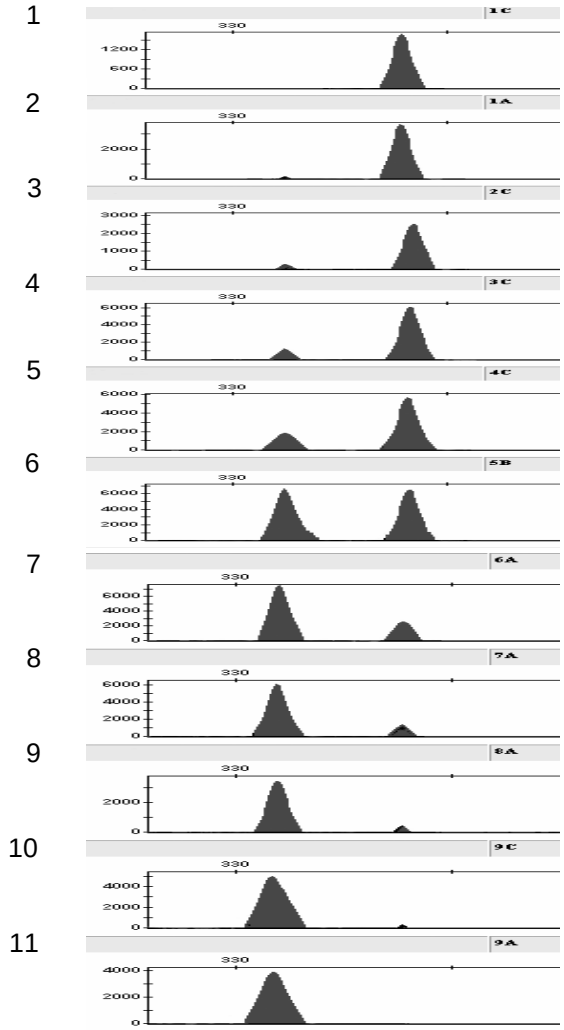

Fig. 1. Results of the expression of *Mre11* from A and B genomes after automatic fluorescence analysis of one of the three replicates for each of the 9 dilutions tested. Controls of the expression of the A and B genome genes were included (1 and 11 respectively): 2 = dilution 1:100; 3 = 1:20; 4 = 1:7; 5 = 1:3; 6 = 1:1; 7 = 3:1; 8 = 7:1; 9 = 20:1; 10 = 100:1.

Table 1. Expression values of *Mre11* belonging to the A and B genomes for the 9 dilutions tested, and the confidence intervals obtained

Known dilution ratio	Fractional expected ratio	Observed signal ratio (± SD)	95% confidence interval
1:100	0.010	0.016 ± 0.010	-0.010-0.042
1:20	0.048	0.030 ± 0.014	0.006-0.067
1:7	0.125	0.112 ± 0.004	0.101-0.124
1:3	0.250	0.237 ± 0.008	0.215-0.259
1:1	0.500	0.492 ± 0.008	0.471-0.514
3:1	0.750	0.755 ± 0.004	0.743-0.766
7:1	0.875	0.875 ± 0.002	0.869-0.881
20:1	0.952	0.952 ± 0.001	0.949-0.956
100:1	0.990	0.987 ± 0.003	0.979-0.996

Fluorescence SSCP was then used to analyse expression of the transcripts coded by the *Mre11* genes of A and B genomes in the tetraploid *Triticum turgidum* (AABB) using the diploid species

Triticum monococcum (AA) and *Aegilops tauschii* (DD) as controls. Three independent RT-PCR and labelling reactions were performed for each. Again, the fluorescence technique discriminated two different peaks belonging to the A and B genomes (Fig. 2). The possibility of distinguishing more transcripts was demonstrated in *T. aestivum* (AABBDD). In this case, three different peaks were clearly resolved corresponding to the three genomes (Fig. 2).

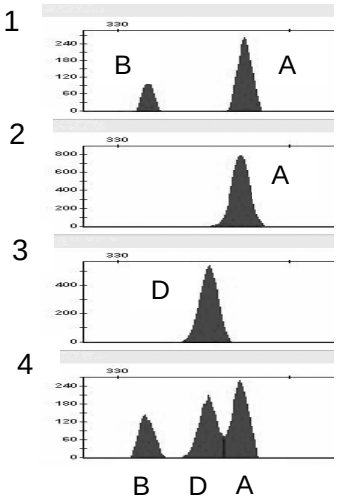

Fig. 2. Results of the fluorescence SSCP analysis of Mre11 performed with *T. turgidum* (1), *T. monoccocum* (2), *Ae. tauschii* (3) and *T. aestivum* (4). Transcripts from the A, B and D genomes were identified.

The fluorescence SSCP technique described here has all the advantages of the radioactive method (Cronn and Adams, 2003) for the study of the expression of homoeologous genes in polyploid species. However, the results obtained are more reproducible, more accurate, and the process is less time-consuming. Moreover, this technique eliminates the risks associated with the use of radioactive compounds, as well as the need for acrylamide gels. In addition, the results are obtained automatically in less than an hour, thus avoiding lengthy exposure times (sometimes several days) and obviating the need to measure band intensities. The technique is a powerful tool for resolving the expression of homoeologous genes not only in tetraploid but also in hexaploid species, and perhaps with species of more complex genomic constitution.

Acknowledgements

The authors would like to thank the MCYT (Ministerio de Ciencia y Tecnología) of Spain for its financial support of this work (Grant No. AGL2003-08128-C02). We thank Pilar Rubio for technical assistance with the fluorescence analysis. Alfredo De Bustos is supported by the Ramón y Cajal Programme of the MCYT.

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