Cloning and use of low copy sequence genomic DNA for RFLP analysis of somaclones in mustard (Brassica juncea (L.) Czern and Coss)

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Brassica juncea (L.) Czern and Coss genomic DNA fragments of 0.5 to 2.5 kb size, obtained by Pst I digestion were cloned into the pUC18 plasmid vector. Screening of the partial library by dot blot hybridization using labelled total genomic DNA as probe revealed that about 45% of the clones carry low copy number DNA sequences. Using some of these clones as probes, DNA polymorphism could be detected among somaclones of B. juncea cv. Varuna.

Restriction fragment length polymorphism (RFLP) analysis is used for tagging genes controlling complex quantitative traits, varietal identification and establishing phylogenetic relationship. Successful application of the technique, however, requires a large number of low copy DNA sequences, used as probes in Southern hybridization. To meet this need, partial libraries of random low copy genomic DNA sequences have been constructed in several plant species\(^1\)\(^-\)\(^4\). The methylation-sensitive Pst I restriction enzyme has played a key role in this context. Pst I can specifically access the low copy sequences which are generally un- or undermethylated. Figdorc et al.\(^5\) reported that more than 75% of Pst I-generated clones carry low copy sequences in the diploid Brassica species — B. oleracea and B. campestris. In this communication, we report construction of a partial Pst I genomic library of low copy sequence genomic DNA and its application in RFLP analysis of somaclones in the alloplloid Brassica juncea (L.) Czern and Coss.

Genomic DNA isolated from 4-day-old etiolated seedlings of B. juncea cv. Varuna, following Dellaporta et al.\(^6\), was purified by CsCl density gradient centrifugation, digested with Pst I and electrophoresed on a 1% agarose gel. DNA fragments, 0.5 to 2.5 kb in size, were electroeluted from the gel and purified by phenol-chloroform extraction.\(^7\) Though the DNA was completely digestable with methylation-insensitive enzymes such as Hind III and Msp I, it was observed that even after overnight digestion with Pst I, a major portion of genomic DNA remained uncut, suggesting that B. juncea genome is highly C-methylated. This resulted in very low yield (3.6 \(\mu\)g out of 40 \(\mu\)g of DNA digested) of desired fragments. Besides, five fluorescing bands seen (Figure 1) within the desired size range indicated that some repetitive DNA sequences are still present in the eluted fraction.
DNA fragments, thus obtained, were cloned in plasmid pUC18. For this, the plasmid DNA was linearized by Pst I and treated with alkaline phosphatase to prevent self-ligation. The vector and the eluted DNA fragments were mixed in molar ratios of 1:4, 1:6 and 1:8 for overnight ligation at 15°C. Competent cells of Escherichia coli JM107 transformed by each of these ligated DNA samples, yielded a total of 92, 148 and 261 recombinant clones respectively. Pst I restriction analysis of 25 randomly chosen clones confirmed the presence of cloned DNA.

For identifying low copy clones, plasmid DNA isolated from all the 501 clones was dot blotted onto nitrocellulose and probed with nick translated α-32P labelled total genomic DNA. In the autoradiograms (Figure 2), the extent of hybridization in different clones, as revealed by visual observations and laser beam densitometric scanning, was found to vary. This variation could be due to the fact that eukaryotic genomes contain about 50% or more of repetitive DNA. Therefore, the amount of α-32P dNTP incorporated in the repetitive sequences during nick translation of total genomic DNA and the extent of its hybridization with the dot blotted recombinant plasmids are expected to be higher than the single/low copy sequences. Consequently, the clones showing very high hybridization signal intensity are likely to be highly repetitive, while those having intermediate and low or as much signal intensity as the control pUC18 are likely to be moderately repetitive and single/low copy respectively. Based on this rationale, 34 (6.8%) clones were identified as highly repetitive, 239 (47.7%) as moderately repetitive and 228 (45.5%) as low copy. This proportion of low copy clones is, however, lower than that reported by Figdore et al.5 in the diploid Brassica oleracea ssp. capitata (76.4% low copy of a total of 161 clones) and in B. campestris ssp. pekinensis (75% of a total of 140 clones).

The amphidiploid nature of Brassica juncea might explain low proportion of low copy sequence clones in our study. Recent reports on hybridization of RFLP markers with B. oleracea monosomic alien chromosome addition lines have indicated occurrence of extensive intragenomic DNA duplications. Besides, many of the low copy sequence clones from the Pst I library of B. oleracea ssp capitata have been shown to hybridize with multiple DNA fragments at intraspecific as well as interspecific levels. It is possible that a low copy DNA sequence from a diploid which is accessible to Pst I, will behave as multicopy or be moderately repetitive in an amphidiploid, depending on the extent of intra and inter-genomic DNA duplications. Presence of such sequences in the amphidiploid would obviously result in low proportion of low copy sequence clones in the Pst I library.

A set of low copy clones thus obtained was employed for assessing DNA polymorphism in the parental variety Varuna and its six somaclones identified phenotypically in replicated trials as early maturing-1, early maturing-2, late maturing, tall-1, tall-2 and productive, in their selfed R0 generation. For this, genomic DNA was isolated following Saghai-Maroof et al.13 with the modification that instead of 15 volumes of 1 × CTAB extraction buffer, 5 volumes of 2 × buffer was used for fresh leaf material, ground in liquid nitrogen. This
protocol was used to eliminate polysaccharides generally known to affect the quality and thus digestability of DNA. On further purification by CsCl density gradient centrifugation, DNA was digested with Hind III, Eco RI and Eco RV (Promega), electrophoresed and transferred onto Gene-Screen plus nylon membrane (DuPont) using an LKB vacuum blotting unit. Hybridization was carried out following Kochert et al. using α-32P labelled recombinant plasmids as probes. Out of 15 probe-enzyme combinations tried 10 combinations detected polymorphism. Clone BJG 431 detected DNA polymorphism among all the selected somacles (Figure 3). The two early maturing somacles could not be distinguished from each other in spite of their phenotypic differences but all the six could be differentiated from the parent variety. The variation in Southern hybridization pattern indicated occurrence of DNA rearrangements and/or point mutations in the selected somacles. These somacles are in the

advanced generation of selfing and have attained uniformity for the selected traits. The observed DNA alterations, therefore, represent stable and heritable somaclonal variation at molecular level.


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