ABSTRACT

Recent advances in molecular biology led to the development of new techniques, such as DNA based molecular markers. This creates opportunities for plant genetic researchers to assess and characterize genetic variation, which has been the foundation for the selection of desirable qualitative and quantitative traits of agronomical importance. Among the techniques, AFLP is one of the most powerful technologies that could identify a large number of polymorphic loci without prior knowledge of DNA sequences of the genome and has been widely used for genetic fingerprinting, genome mapping and genetic variability studies.

Cultivated tea belongs to the genus *Camellia* and it is a diploid with basic chromosome number 15. It is an important non-alcoholic beverage and a ‘health drink’ which is widely consumed all over the world. Secondary metabolites, particularly flavonols and polyphenols (catechins), are of great abundance and contribute to their flavour and health properties. For major tea-producing countries, tea is a major source of foreign currency revenue. So, to meet the high demands of this plantation crop, there is a continuous need for its genetic improvement by application of genetic markers. Tea has long gestation period and is an outcrossing species. Therefore, the conventional methods of breeding take a long time to develop genetically improved plant with desirable traits. Clonal selection is an important and widely adopted method of tea improvement as they are genetically uniform and give uniform yield and quality. In recent years, efforts have been given worldwide, integrating the DNA marker based technologies on genetic improvement of tea.
The development of genetic linkage maps using DNA based markers is a new plant breeding tool that functions as a valuable reference for locating important genes on the maps that allow marker assisted selection of agronomically important traits. Till date, six DNA marker based linkage maps have been developed using different markers such as RAPD (Randomly Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), ISSR (Inter Simple Sequence Repeats), CAPS (Cleaved Amplified Polymorphic Sequences), SSRs (Simple Sequence Repeats). STS (Sequence Tag Sites) markers in different genetic backgrounds (Tanaka 1996. Hackett et al. 2000. Huang et al. 2005. Huang et al. 2006, Kamunya et al. 2010 and Taniguchi et al. 2012). A molecular linkage map is not only a prerequisite for linking a QTL, it is essential to map them for efficient selection and subsequent transfer of the trait. Therefore, to achieve the ultimate goal of genetic improvement of tea, further efforts are required to construct a high density map and to locate the quantitative trait loci (QTL) and other important agronomic traits. Although the density of integrated genetic maps can be extremely high, it may still not be sufficient to tag a specific gene and more maps would help in filling the existing gaps in the present maps.

Tea genome size is estimated to be $4.0 \times 10^9$ bp (Tanaka et al. 2006), so AFLP (Vos et al. 1995). Therefore conventional AFLP based marker analyses would be cumbersome due to generation of large number of bands. So, a modification of conventional AFLP, TE-AFLP (Three Endonuclease-AFLP) method (van der Wurff et al. 2000) that provides high discriminatory power and reduction in the number of
bands would be suitable for tea. Negi et al. (2005) also concluded that TE-AFLP was the best technique for fingerprinting of tea.

For the visualization of AFLP products in polyacrylamide gels, silver staining revealed similar sensitivity and resolution with the autoradiography, reduced the time, cost and eliminated hazard of working with radioisotopes (Vantoai et al. 1996, Chalhoub et al. 1997). In addition, recovery of fragments from the dried gels made silver staining a more useful and versatile detection method.

Therefore, there is a strong case for cheap, reliable, informative silver stained TE-AFLP marker studies on a mapping population of tea for obtaining a possible genetic linkage map and if possible to locate some traits that are related to tea production.

With this background, the present study has the following objectives:

1. Identification of TE-AFLP markers using bi-clonal seed stocks which are maintained at Tea Research Association (TRA), Tocklai, Jorhat, India.
2. Construction of a genetic linkage map based on the above TE-AFLP markers.
3. Nucleotide sequencing and analysis of selected TE-AFLP based amplicons.

In order to achieve these objectives the following approach was followed:

1. Mapping population consisting of 117 genotypes of the F₁ population of the bi-clonal seed stock TS 463, through a cross between two TV clones i.e. TV₁, an Assam-China hybrid with another Cambod clone, TV₁₉ were
collected from Tea Research Association (TRA), Tocklai, Jorhat, Assam, India.

2. Genomic DNA extraction was done using modified CTAB method, especially designed for plants containing high polysaccharide and polyphenolic components (Porebski et al. 1997).

3. TE-AFLP analysis was performed with minor modifications according to the protocol described by van der Wurff et al. (2000).

4. Following TE-AFLP amplification reactions, 6% denaturing polyacrylamide gel electrophoresis were done.

5. After the electrophoresis, silver staining was carried out to visualize the DNA fragments in the sequencing gel. The procedure for silver staining was based on the procedure of Caetano-Anolles and Gresshoff (1994) with few modifications.

6. Elution of AFLP fragments was done according to the protocol described by Ausubel et al. (1997) and Chen and Ruffner (1996) with minor modifications.

7. PCR products with only the specific target band were also purified using ethanol precipitation as described by Huang and Cloutier (2007).

8. Purified PCR products were sequenced based on the dideoxy chain termination method of Sanger et al. (1977).

9. Binary data obtained for the TE-AFLP profiles were analysed using four statistical parameters i.e. PIC (Polymorphic Information Content), Marker index (MI), Effective multiplex ratio (EMR) and Resolving power (Rp) for
evaluating the discriminating power and the information content of the selected 5 primer combinations.

10. Linkage analysis was performed using all polymorphic markers by observing parental segregation patterns separately using JoinMap 4.0 software (van Ooijen 2006).

11. The correlation between the number of AFLP markers and the length (size) of the linkage groups was analysed using the Pearson correlation coefficient.

12. Two methods were used to estimate genome length: Fishman et al. (2001) and method 4 as described by Chakravarti et al. (1991).

13. Nucleotide sequences obtained were queried (BLASTn) individually with three different databases on NCBI GenBank (National Center for Biotechnology Information).

14. Open Reading Frame (ORF) for some selected sequences were determined using ORF finder (NCBI).

The results obtained in the present study can be summarised as follows:

1. A ‘narrow-down’ strategy for the primer combination was beneficial in choosing the optimised TE-AFLP primer combinations. At the final step, 5 out of 36 primer combinations which were highly polymorphic in the tea sample collected were chosen for further studies.

2. There was a lack of correlation among the statistical parameters taken for our study i.e. PIC, MI and Rp indicating that a single parameter was not sufficient to assess the informativeness of a primer combination.
3. Genetic load and the scoring error in AFLP were likely the contributing factors to the segregation distortion observed in our study. However, the exact factor is not known.

4. The map of the TV1 parent consisted of 36 markers in ten linkage groups while TV19 parent consisted of 30 markers in eleven linkage groups. The number of linkage groups obtained in this study is less than the haploid chromosome number ($n=15$). This discrepancy in the number is due to the low density of markers.

5. Observed genome lengths were calculated as 388.9 cM and 410.7 cM for TV1 and TV19 maps respectively.

6. The observed genome coverage was determined as 50.76% and 45.91% for TV1 and TV19 respectively.

7. The existence of minor linkage groups and unlinked markers (25 markers in TV1 parent and 9 markers in TV19 parent) indicates that there are many large gaps with few markers.

8. The large intervals (> 20cM), observed in both the maps is due to the low-density of the maps obtained.

9. In our study, the bridging marker are clustered among them and not linked with any markers specific to either parents (1:1 markers) which was uninformative for merging linkage groups and thus no integrated map could be developed.

10. The Pearson correlation coefficient ($r = 0.91$ and $r = 0.83$) indicated that there was a highly significant positive correlation between the number of markers and linkage group size. This supports the contention that the
distribution of AFLP markers was relatively even in linkage groups of IV1 and TV19 map.

11. BLAST analyses showed that the AFLP marker sequences for functional genes appeared to be relatively less compared to the non-coding regions.

12. However, some sequences showed homology with unigenes which were related to nitrogen assimilation, drought, pathogen resistance, heavy-metal stress tolerance, and resistance to biotic and abiotic stresses. The identification of such markers is an important resource for further research aimed at understanding physiological processes important for tea cultivation and quality.
REFERENCES


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