Molecular profiling and development of DNA marker associated with drought tolerance in tea clones growing in Darjeeling

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Amplified Fragment Length Polymorphism (AFLP) fingerprints were developed for 29 Darjeeling-grown tea clones. AFLP diversity estimates based on Jaccard's coefficient allowed separation of the 29 clones into three clusters. Genetic relatedness between the clones was found to be at 70% level.

Random Amplified Polymorphic DNA (RAPD) analysis of DNA of ten short-listed (on the basis of field performance for drought tolerance) clones using 11 primers, revealed 180 PCR products of which 131 were polymorphic bands.

Activity of drought-specific superoxide dismutase (SOD) and ascorbate peroxidase (APX) isozymes was found to be appreciably high in RR17/144, CP1, TV26 and AV2. Regression analysis using peak areas (from scans of stained activity-gel preparation) of Cu-Zn SOD and APX II as dependent variables and RAPD band scores as independent variable revealed that OPAH02 primed DNA band at 1400 bp was associated with high activity of the drought tolerance-specific isozymes. Using Fisher's exact test (*F*-test), this association was found to be at 99.9% confidence level.

CULTIVATED tea belongs to the genus *Camellia* and consists of three species each with a specific plant type[1], viz. *Camellia sinensis* (China type), *Camellia assamica* (Assam type) and *Camellia assamica lasiocalyx* (Cambod type). Among these, *C. sinensis* was the earliest known tea plant that was used as a beverage. Although belonging to the single genus *Camellia*, natural cross-pollination between these species and subspecies has resulted in considerable variation between individual tea plants. Selection of plants with desirable traits from variations generated through natural/artificial crossing[2] or somaclonal variation[3] followed by vegetative (clonal) propagation of these perennial plants constitute the cultivation practice of the tea industry.

Tea plants growing in Darjeeling are unique in their association with a typical eco-geographical habitat. A study of molecular profiles to obtain DNA fingerprints in order to establish the molecular identity of tea clones for documenting the germplasm seems a worthwhile endeavour. Such DNA fingerprinting pattern would also help monitor genetic stability in aged tea clones and also fidelity in tissue culture-derived planting material[4,5]. Correlation of genomic variation with expressed parameter for desirable traits in order to establish trait-associated DNA markers for germplasm screening would repay careful investigation.

A relatively recent method of PCR-mediated DNA amplification-associated genome analysis focusing on dominant markers is Amplified Fragment Length Polymorphism (AFLP)[6–9]. This technique permits inspection of polymorphism at a large number of loci within a short period of time and thus is useful in detection of polymorphism between closely related genotypes[10]. Comparison between RFLP and AFLP maps[11] indicates a random distribution of AFLP markers along the genome. In the present study AFLP analysis was undertaken with 29 tea clones growing in Darjeeling, with an aim to developing DNA fingerprints specific for each clone.

Notwithstanding the preference of AFLP analysis for deriving complete molecular profile for germplasm documentation, for simplicity in scoring species-specific DNA bands on DNA fingerprinting patterns, an alternative method of PCR-based DNA analysis, (viz. Random Amplified Polymorphic DNA (RAPD) method) is often used. RAPD markers have often been used for studying genetic diversity within plant germplasm collections[12,13] and have been proposed by several groups as efficient tools for identification of DNA markers associated with agronomically important traits[14,15]. This is a rapid method that is technically and financially less demanding compared to the AFLP method and thus could be more useful for routine germplasm screening. In view of this, an attempt has been made to use RAPD studies to establish DNA fingerprints that could be correlated with specific drought-related biochemical parameters for identifying DNA fragments associated with drought-tolerant tea clones.

Tea plants are perennial and have long reproductive cycles. Thus for rapid availability of uniform raw material (i.e. fresh tea leaf) vegetatively propagated tea clones constitute the plant population for use in the tea industry. In such populations, linkage study through filial generations is time-consuming. Under such circumstances, developing DNA markers through association study supported by regression analysis between DNA fingerprinting pattern and precise biochemical parameters of phenotypic

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traits in plants belonging to a narrow gene pool, could provide an useful alternative.

Cell-protective enzymes, specifically Cu-Zn cytosolic superoxide dismutase (SOD)[16,17] and ascorbate peroxidase (APX)[18], have been reported to be correlated with drought tolerance in plants. In this study, an attempt has been made to establish DNA markers associated with drought tolerance in tea plants through use of regression analysis involving RAPD pattern and drought-specific isozyme activity.

The objectives of this study were: (i) To establish DNA fingerprints for some Darjeeling-grown tea clones with an aim to developing molecular profiles of these clones and for studying clustering and phenotypic diversity among individual clones using AFLP analysis. (ii) To establish specific DNA markers associated with drought tolerance in tea plants through regression analysis using RAPD banding pattern and drought tolerance-associated SOD and APX isozyme activities.

Materials and methods

Leaves of plants from individual clones were collected from Ging Tea Estate, Darjeeling and stored at – 80°C for DNA studies. For the enzyme studies, freshly detached tea leaves were used for experiments within 24 h.

Genomic DNA extraction

Two hundred milligrams of frozen young leaves were used for DNA isolation. The CTAB (cetyltrimethylammonium bromide) method of Gawel and Jarret[19] was employed to extract DNA and on an average 20 µg of DNA was obtained. After agarose gel electrophoresis, DNA was calibrated on the basis of ethidium bromide fluorescence using defined amount of DNA as standard.

AFLP analysis

AFLP analysis of 29 Darjeeling-grown tea clones was carried out using GIBCO-BRL AFLP analysis system-II kit. The extracted genomic DNA was digested with *Eco*RI and *Mse*I followed by ligation with *Eco*RI and *Mse*I adapters at 20°C. The ligated DNA fragments were preamplified using *Eco*RI + '0 selective nucleotide' and *Mse*I + '1 selective nucleotide' primer. Primers with two selective nucleotides for *Eco*RI primer and three selective nucleotides for *Mse*I primer at the 3′ end were employed for selective amplification; the *Eco*RI primer was end-labelled with γP^{33} ATP. PCR was conducted using 65°C as the initial annealing temperature for the first cycle. For the subsequent 11 cycles, the annealing temperature was successively reduced by 0.7°C. Twenty-three cycles were run at 56°C annealing temperature. PCR products were run on 6% denaturing polyacrylamide gel in 1X TBE buffer in a sequencing gel system (BIO-RAD) and transferred to filter paper (Whatman). The filter paper containing the gel was vacuum-dried and then exposed to Kodak X-ray film. A total of eight primer pair combinations were used. A representative photograph of AFLP banding pattern is shown in Figure 1. Total number of AFLP bands observed in the 29 clones studied, number of monomorphic/polymorphic bands present, and percentage of polymorphic bands obtained are given in Table 1.

Data scoring and analysis

The PCR-generated bands produced by each primer pair were scored as present and absent on autorads and the data generated with eight primer pairs were used to compile a binary matrix for cluster analysis. Genetic similarity among tea clones was calculated using Jaccard's similarity coefficient[20]. This similarity matrix was used to perform a cluster analysis using the unweighted pair group method with arithmetic averages (UPGMA) that generated a dendogram (Figure 2).

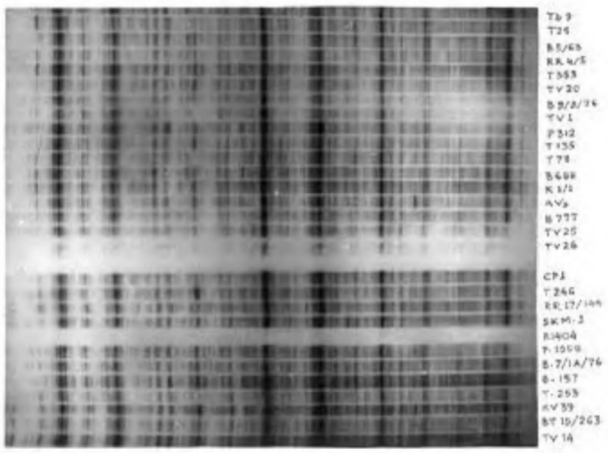

Figure 1. Portion of AFLP fingerprint of 29 elite tea clones using primer pair E_{ag} M_{cta}.

Table 1. AFLP generated among 29 tea clones from Darjeeling region

Primer pair	Total no. of bands	No. of polymorphic bands	Percentage of polymorphism
E_{at} M_{ctt}	121	82	67.76
E_{ta} M_{cat}	37	19	51.35
E_{tg} M_{cta}	99	76	76.76
E_{tc} M_{cac}	111	79	71.17
E_{ag} M_{cta}	96	66	68.75
E_{ac} M_{cta}	48	36	75.00
E_{aa} M_{caa}	78	45	57.69
E_{tg} M_{ctg}	87	66	75.86
Total	677	469	68.04

The degree of AFLP polymorphism was calculated using Shannon's diversity index for phenotypic diversity[21]. Calculated values are presented in Table 2.

$$H_s = \sum f_i \ln f_i,$$

where H_s is the phenotypic diversity and f_i the frequency of phenotype. The average group diversity over three groups of tea clones $(H_{avg}) = {}^1/_n \sum H_s$, where n is the number of groups of the tea clonal population. Thus phenotypic diversity of clones from all groups, $H_w = -\sum f \ln f$, where f is the frequency of AFLP in all the three groups. Thus, within group diversity of tea population = H_{avg}/H_w. Between group diversity of tea population = $H_w - H_{avg}/H_w$.

RAPD studies

For RAPD analysis, DNA of young leaves from each clone was used as template in the PCR reaction mixture of total volume 25 µl, containing 25 nm dNTPs, 40 ng primer and 0.3 µl *Taq* DNA polymerase. The reaction was allowed for 45 cycles under 36°C annealing temperature. PCR products obtained using eleven random decamer primers such as OPAB06, OPAB18, OPAH18, OPAH02, OPAB17, OPAC19, OPAH12, OPAL04, OPAA02, OPAG13 and OPAL08, were separated on 1.2% agarose gel and visualized over UV light in the presence of ethidium bromide. RAPD analysis of randomly selected plants within each clonal population confirmed the genetic uniformity of plants within each clonal plot (data not presented). For use in the present study, a comparative analysis of ten shortlisted (on the basis of drought tolerance/susceptibility) clones was undertaken. This RAPD study was repeated (for each set of ten clones) with leaves collected from randomly selected plants within each clonal plot. Representative gel image of RAPD pattern is shown in Figure 3 *a*. Figure 3 *b* shows the RAPD pattern with the same primer on genomes of four clones (that are also present in Figure 3 *a*) that exhibit drought tolerance in the field (Figure 3 *b* was not used for scoring RAPD bands).

Primers used in the RAPD study, total number of RAPD bands observed through all the ten clones, total number of monomophic/polymorphic bands present, and percentage of polymorphic bands obtained are given in Table 3.

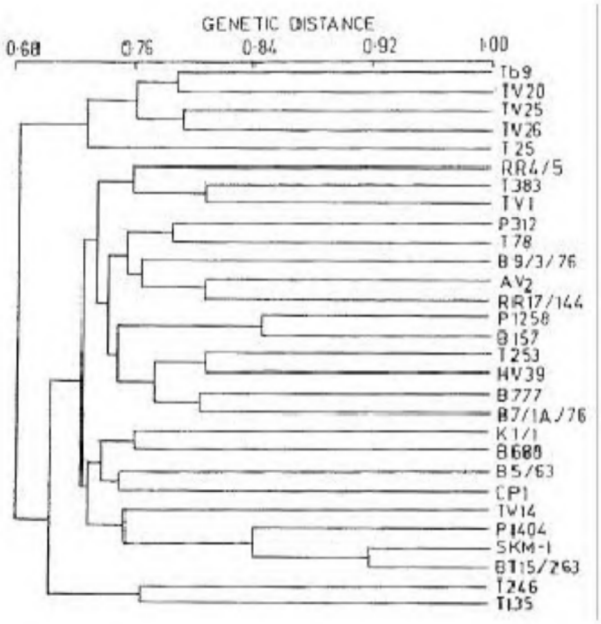

Figure 2. Dendrogram of 29 tea clones using eight primer pair combinations based on UPGMA cluster analysis of AFLP data.

Table 2. Partitioning of genetic diversity into within (H_{avg}/H_w) and between ($H_w - H_{avg}/H_w$) populations of tea clones for eight primer pair combinations

Primer	H_w	H_{avg}	H_{avg}/H_w	$H_w - H_{avg}/H_w$
E_{at} M_{ctt}	2.92	2.00	0.68	0.31
E_{ta} M_{cat}	2.95	1.95	0.66	0.33
E_{tg} M_{cta}	3.19	1.92	0.60	0.39
E_{tc} M_{cac}	3.22	2.00	0.62	0.37
E_{ag} M_{cta}	3.25	2.01	0.61	0.38
E_{ac} M_{cta}	3.14	1.99	0.63	0.36
E_{aa} M_{caa}	3.31	2.05	0.62	0.37
E_{tg} M_{ctg}	3.04	1.95	0.65	0.34
Mean (χ)	3.12	1.97	0.63	0.36

Stress-related studies

Ten tea clones shortlisted for drought tolerance/susceptibility on the basis of pot experiments were used for RAPD analysis and isozyme studies.

Stress-related isozyme activity

Detached (water-stressed), young tea leaves were extracted in extraction buffer and the extracts were run on non-denaturing gels. Activity of enzymes was studied by allowing the reaction of iso-enzyme in gels by immersing the gel in appropriate reaction mixture, followed by stopping enzyme activity by immensing gel in appropriate staining solution.

SOD and APX activity

Isolated leaves were homogenized in 50 mM pottasium phosphate (pH 7.8) buffer for SOD (EC 1.15.1.1) and in 100 mM sodium phosphate (pH 7.8) buffer for APX (EC 1.11.1.11). Enzyme activities in the extract were visualized on non-denaturing activity gel (12% polyacrylamide) after electrophoretic separation followed by immersing the gel in appropriate staining solution.

Elecrophoretically separated SOD isozymes were visualized by reduction of Nitro Blue Tetrazolium (NBT) by O_2^- generated in a reaction between TEMED and Ribofla-

vin[22]. Bands representing the isozymes remain colourless due to the absence of O_2^- that had been scavenged by isozyme activity; rest of the gel was stained blue due to reduced NBT. Desitometric scans (at 632.8 nm) of negatively stained isozyme activity bands are shown in Figure 4 *a*.

The APX activity gel staining was similar excepting that instead of riboflavin, H_2O_2 and Ascorbate were used[23] to generate O_2^-. Negative staining of isozyme bands is documented in a gel doc apparatus (Image Master VDS, Pharmacia Biotech). The negatively stained isozome activity bands scanned at 632.8 nm; a representative picture of the densitometric scans is shown in Figure 4 *b*.

Correlative study between isozyme activity and DNA fingerprint to develop specific DNA markers for drought tolerance using regression analysis

Regression analysis was conducted using data of isozyme study (of SOD and APX) and RAPD bands. Association of RAPD markers with SOD and APX activities was investigated using multiple regression analysis approach. Enzyme activities, viz. of appropriate SOD and APX isozymes (peak areas) were treated as dependent variables and the various RAPD bands (scored as 1 for presence of band and 0 for absence) were treated as independent variables. The regression analysis was based on the model:

$$Y = a + b_1m_1 + b_2m_2 + \ldots + b_jm_j + \ldots + b_nm_n + d,$$

where Y is the enzymatic trait, m the RAPD marker, b the partial regression coefficient, d the between accession residual which is left after regression. Extent of association between the RAPD bands and Cu-Zn SOD and chloroplastic APX activities is shown in Table 4.

Results and discussion

AFLP analysis of genomes has been found to provide useful molecular profiles of closely related asexually propagated plants[24,25]. This method was used to analyse genomes and for evaluating genetic diversity of 29 Darjeeling-grown tea clones.

A representative photograph of AFLP of 29-tea clones is given in Figure 1. Using eight primer pair combinations in the 29 clones 677 PCR products, including 469 polymorphic bands were observed (Table 1). The number of polymorphic fragments amplified by individual primer pairs ranged from 51.35 (with primer pair E_{ta} M_{cat}) to 76.76% (with primer pair E_{tg} M_{cta}). The calculated average percentage of polymorphic fragments per primer pair was 68.04. DNA bands on the fingerprint were scored for presence (+ 1) and absence (+ 0) and estimates of similarity between genotypes were determined based on the probability that an amplified fragment from one accession will also be present in another[26]. To determine the level of relatedness among these genotypes, a similarity matrix was constructed with AFLP as a phenetic marker, assuming that the presence or absence of a discrete character in two or more genotypes results from the same genetic changes[27]. Simple matching coefficient was used for cluster

Table 3. Sequence of 11 random primers with the number of scorable amplified and polymorphic bands

Primer	Sequence of primer (5′–3′)	Number of amplified bands	Number of polymorphic bands	Percentage of polymorphic band
OPAA02	GAGACCAGAC	11	5	45.45
OPAB06	GTGGCTTGGA	15	10	66.66
OPAB17	TCGCATCCAG	16	8	50.00
OPAB18	CTGGCGTGTC	16	10	62.50
OPAC19	AGTCCGCCTG	21	20	95.24
OPAH02	CACTTCCGCT	15	14	93.33
OPAH12	CACTTCCGCT	14	11	78.57
OPAH18	GGGCTAGTTA	24	22	91.66
OPAG13	GGCTTGGCGA	14	5	35.71
OPAL08	GTCGCCCTCA	19	17	89.47
OPAL04	ACAACGGTCC	15	9	60.00
Total		180	131	69.87

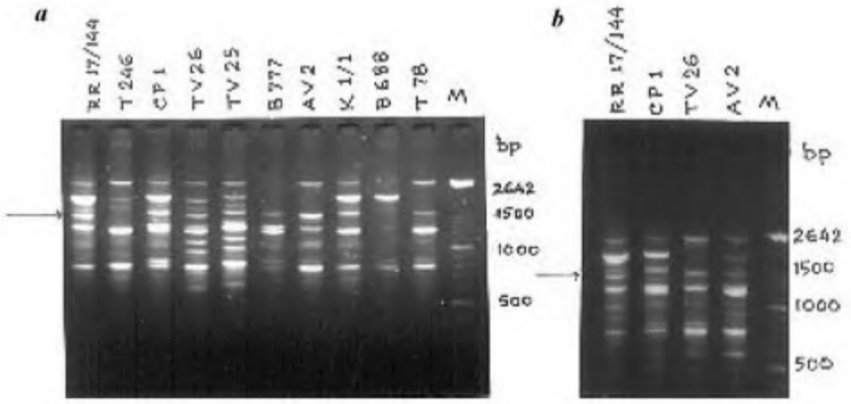

Figure 3 ***a***, ***b***. RAPD pattern of tea germplasm using primer OPAH 02. DNA fragment (1400 bp) marked by '→'. M, 100 bp mol. wt marker.

analysis by UPGMA. The dendogram thus derived indicates a clear division into three groups (Figure 2). Similarity level up to 50% in cluster analysis is indicative of plants derived from interspecific hybridization[28]. In this study, similarity between the plants studied ranges between 68 and 92%. This indicates that the Darjeeling-grown tea plants studied here belong to a narrow gene pool originating through intra as well as inter-specific hybridization. From morphology-based parameters of tea plants, Wight[29] also concluded that Indian tea clones have originated from inter-and intra specific hybridization. A study on AFLP analysis of tea genomes for determining the relationship between Indian tea clones (from parts of India other than Darjeeling), including a large number of *C. assamica* types and Kenyan tea clones have revealed[30] about 50% similarity, suggesting that those clones have originated mainly through interspecific hybridization. Shannon's index of phenotypic diversity was used to partition the diversity into within and between population components. An assessment of the proportion of average diversity within populations (H_{avg}/H_w) was found to be 63%, while that between populations (H_w–H_{avg}/H_w) was found to be 36% (Table 2). These results may be compared with the study on Indian and Kenyan tea clones that report 58% average genetic diversity within the tea population and 42% diversity between the three types of clonal populations[30].

AFLP study allows extensive inspection of genomes within a short span of time and thus provides comprehensive information on genetic relatedness between plants; RAPD analysis has also been shown to be an efficient tool for precise characterization of genetic diversity[31] between closely related genotypes[32], such as the tea plants studied here. Use of RAPD analysis as a useful means of analysing tea genomes has also been reported earlier[33]. In the present study, RAPD analysis was undertaken with an aim to developing drought tolerance-associated DNA marker.

For the RAPD study presented here some tea clones reported to be drought tolerant/sensitive (on the basis of field performance) were used. Figure 3 *a* and *b* shows RAPD pattern of the tea clones, where the arrow mark indicate a DNA band at about 1400 bp that is present only in some clones, viz. RR17/144, CP1, TV26 and AV2 but not in the others, viz. T246, TV25, B777, K1/1, B688, T78, when primer OPAH02 is used. Reproducibility of data was confirmed after observing identical fingerprinting patterns in three repeat experiments. A similar approach in demonstrating reproducibility in RAPD studies have also been reported earlier[34]. Use of eleven primers in RAPD studies on ten clones revealed 180 PCR bands; among these 131 bands (i.e. 69.87% of total 180 amplified fragments) were polymorphic (Table 3). This shows that the RAPD method is capable of revealing appreciable levels of polymorphism in tea clones.

Polymorphic marker generated from the fingerprinting pattern of plants belonging to a narrow gene pool represents independent characters for specific traits[35]. In view of the genomic polymorphism observed within the narrow gene pool of the plants studied here, an attempt was made to develop drought tolerance-associated molecular (DNA) marker(s). For this, a study was made between easily scorable RAPD (dominant) markers and biochemical parameters of drought tolerance in plants.

Activity of stress-related cell-protective enzymes has been reviewed at length[36,37]. Cytosolic Cu–Zn SOD[38] and cytosolic APX[39] are known to be associated with drought stress in plants. Isozyme marker-based assessment for drought tolerance in the clones under study was made by assessing activity of SOD and APX isozymes. Densitometric scan of activity gel of SOD (Figure 4 *a*) shows four major peaks; the peak at about 29 kDa (band IV) represents the Cu–Zn SOD[40]. This peak was found to show higher activity (peak area above 100 mm^2) in clones RR17/144, CP1, TV26 and AV2 compared to the area under peak IV in T246, TV25, B777, K1/1, B688, T78 (peak area below 70 mm^2). This suggests that clones RR17/144, CP1, TV26 and AV2 possess drought-tolerant trait, thus confirming the preliminary observations made in field experiments.

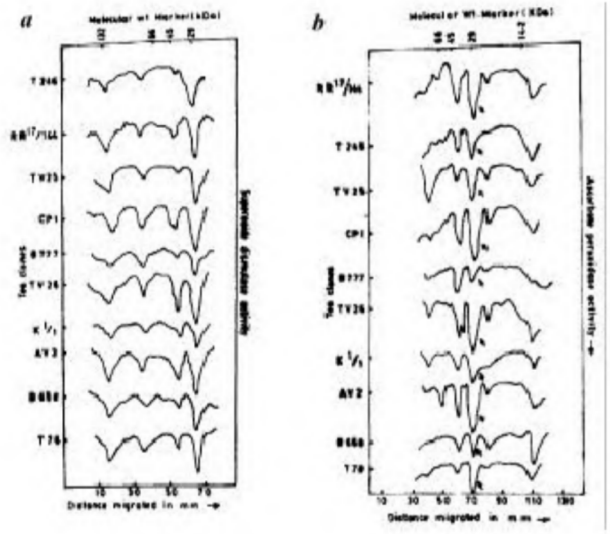

Figure 4. Densitometric scans of (*a*) SOD and (*b*) APX isozymes from ten clones run on 12% native polyacrylamide gel.

Table 4. Association between Cu-Zn SOD, APX II and RAPD band at 1400 bp

Tea clone	Cu-Zn activity peak area (mm^2)	APX II activity peak area (mm^2)	Association with drought-related isozyme activity
RR17/144	104	91	RAPD bands observed
CP1	121	110	at 1400 bp using
TV26	122	93	OPAH02 primer
AV2	120	97	(significant, $P < 0.001$)
T246	74	33	No specifically significant band observed

Cultivars of *Nicotiana tabacum* with highest drought tolerance show highest ascorbate peroxidase activity[18]. Polyacryamide gel electrophoresis study[41] of tea leaf extract revealed two isozymes; one of these, namely APX II was found to be a monomer with molecular weight of 34 kDa. Our study of APX isozymes (Figure 4 *b*) shows two major bands representing activity of APX isozymes as detected by NBT staining of gels. In densitometric scans of the activity gels, the two bands are represented as two peaks at about 40 kDa (APX band I) and 29 kDa (APX band II); (Figure 4 *b*). The isozyme peak at about 29 kDa (APX II) exhibited particularly high activity in clones RR17/144, CP1, TV26, AV2 (peak area above 90 mm^2); T246, TV25, B777, K1/1, B688, T78 exhibited lower activity (peak area below 50 mm^2). It may be noted that the clones that showed high APXII activity (Figure 4 *b*) also showed high Cu-Zn SOD activity (Figure 4 *a*). These clones also appear to be drought tolerant from field performance data. The amplified fragment at 1400 bp was found to be present in these clones only but not in the others (Figure 3 *a* and *b*).

Association studies between RAPD bands and traits of interest have been reported[42]. For precision in establishment of trait-related DNA markers, Virk *et al.*[43] have used RAPD analysis of highly diverse accessions of rice in multiple regression analysis to determine associations between the DNA marker(s) and quantitative traits. In the present study, regression analysis (Table 4) was undertaken to determine the association of specific isozyme activity (Figure 4 *a* and *b*) peak areas as dependent variables and RAPD band(s) (Figure 3 *a*) score as independent variables. Step-wise regression showed that the RAPD band (at 1400 bp) obtained with OPAH02 primer has a highly significant regression coefficient for Cu-Zn SOD activity ($b = 0.970$) and APX II activity ($b = 0.968$). Using Fisher's exact test (F-test), the association between Cu-Zn SOD and APX II, and the RAPD band of 1400 bp was found to be significant at 99.9% confidence. Being associated with clones exhibiting high activity of drought tolerance-specific isozymes, this DNA band (marker) could be used in germplasm screening for drought tolerance in tea plants.

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