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Genetic basis of catechin content in some selected clones of Tea [*Camellia sinensis* (L.) Kuntze]

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Abstract

Genetic diversity of five selected clones (seed stocks TS656, TS657, and TS658) of *Camellia sinensis* L. was studied using CAPS (PCR-RFLP) marker. For CAPS analysis of *PAL* exon2, restriction enzyme *Kpn*I and for *CHS*2 exon2 restriction enzymes, *Rsa*I and *Rca*I were used. Genomic DNA was amplified by PCR using primer pairs targeting exon2 of both *CHS*2 and *PAL* genes. Two types of profiles were observed from the restriction profiles obtained for exon2 of *CHS*2 digested by *Rsa*I. Characteristic DNA patterns obtained by digesting the *CHS*2 exon2 amplicon with *Rca*I showed two different allelic forms because only one allele contained a restriction site for this enzyme. A *Kpn*I restriction site in *PAL* was found to be completely absent in most of the samples except for a few samples. The PCR products of both the targeted genes of some clones were sequenced. The nucleotide sequences showed diversity of the targeted genes in the samples studied. *PAL* and *CHS*2 are directly involved in the secondary metabolite pathway in plants leading to the biosynthesis of many secondary metabolites, including flavonoids.

Key words: Catechin; Chalcone synthase; Cleaved amplified polymorphic sequence; Phenylalanine ammonia-lyase, Polymorphism

INTRODUCTION

Cultivated Tea [*Camellia sinensis* (L.) Kuntze], of Theaceae, is represented by three taxonomic varieties: *C. sinensis* (L.) Kuntze var. *sinensis*; *C. sinensis* var. *assamica* (W.J.Mast.) Kitam., and *C. sinensis* var. *lasiocalyx* (G. Watt) A.P.Das & C.Ghosh (Das & Ghosh 2016). These are also known as China, Assam, and Cambod varieties, respectively (Banerjee 1992). Tea leaves are rich in flavonoids, which are one of the largest classes of plant phenolics occurring abundantly in vegetables, fruits, and green plants (Gershenzon 2002). Catechins constitute the major group of flavonoids found in tea leaves and are responsible for the characteristic taste, aroma, and color of tea. The major catechins in green tea-leaves are epicatechin (EC), epigallocatechin (EGC), epigallocatechin gallate (EGCG), and epicatechin gallate (ECG), all of which are water-soluble polyhydroxylated flavonoids. A high content of total catechin is a good indicator of high quality tea (Anan & Nakagawa 1974; Obanda *et al*. 1997).

The present work was carried out to analyse the genetic diversity of two targeted genes, phenylalanine ammonia-lyase (*PAL*), and chalcone synthase2 (*CHS*2) of five selected clones of *Camellia sinensis* L. using CAPS (PCR-RFLP) marker.

MATERIAL AND METHODS

Plant material

Two biclonal seed stocks, TS379 and TS463, and three clonal seed stocks, TS656, TS657, and TS658, of tea maintained at the Tea Research Association (TRA) in Tocklai, Jorhat, India were selected for the study. Seed stock TS379 was obtained by crossing clones 14.12.16 (China type) and 14.5.35 (China type), and seed stock TS463 was obtained by crossing clones TV1 (Assam-China hybrid) and TV19 (Cambod type). Twenty F_2 progenies each of TS379 and TS463 seed stocks were taken. Twenty clones each of the clonal seed stocks all Assam- China types were also included in the analysis.

DNA isolation

Total DNA was extracted from frozen tissue (1gm) using the CTAB method (Porebski *et al*. 1997) with minor modifications. The collected DNA pellet was suspended in an appropriate quantity of nuclease-free Milli-Q water and stored at -20° C. Quality and concentration of the DNA samples were assessed with agarose gel electrophoresis and by using Lambda 35 UV/VIS Spectrophotometer, Perkin Elmer, MA, USA. The A260/A280 ratio for the DNA samples isolated ranged from 1.8 - 2.0.

Target DNA segments and primer design

Marker development was based on the two genes *PAL* and *CHS*2, for which nucleotide sequence information is available for tea in the global gene databases. The gene segments of exon2 for both *PAL* and *CHS*2 were targeted because exons constitute part of the mature mRNA transcript responsible for the synthesis of the corresponding proteins. Although intron segments tend to harbor greater diversity, we deliberately excluded them, as they are not part of the mature mRNA. For amplifying exon2 of *PAL*, primers (PX2F 5'-AGGCTAACATACTCGCC-3' and PX2R 5'-TGCGATAAGAATTGCAC-3') were designed based on the *C. sinensis* cultivar Yabukita cDNA sequences of *PAL* in GenBank (Accession: D26596) using the DS GENE version 1.1 software. Primers reported by Kaundun and Matsumoto (2003) were used for amplifying exon2 region of *CHS*2.

Polymerase Chain Reaction

The polymerase chain reaction was carried out in a 25-ìL volume containing approx. 50-100 ng genomic DNA, 2 pM primers, 200 ìM dNTP, 2 mM MgCl2, 50 mM KCl, 500 ng BSA, 10 mM Tris-HCl, pH 8.3, and 0.5 U Taq polymerase. PCR was performed in a thermal cycler (Applied Biosystems 9700) programmed for an initial denaturation step of 94° C for 5 min followed by 35 cycles of 1 min at 94° C, 1 min at the annealing temperature, and 1 min at 72°C. A final elongation step of 10 min at 72° C was also included. The PCR products were separated on a 1% agarose gel.

Digestion of PCR products

A total volume of 50 ìL PCR products was digested separately with restriction endonucleases including *Rsa*I, *Rca*I, and *Kpn*I following the respective manufacturer instructions (Roche Diagnostics & New England BioLabs). The digested products were separated on a 4% agarose gel.

Nucleotide sequencing

For *CHS*2 exon2, samples TV1, TV19, 1M, 14.12.16, 14.5.35, 1A and 15A, one clone each from clonal seed stocks TS656, TS657 and TS658 were sequenced. For *PAL* exon2, samples TV1, TV19, 12M, 14.12.16, 14.5.35, 6A, one clone each from clonal seed stocks TS656,

TS657 and TS658 were sequenced. For each sample, both the forward and reverse fragments were sequenced and both the nucleotide sequences were aligned using CLUSTAL W 2.1. The overlapping sequence was taken as the total length of the amplified fragment. The sequences obtained were then analysed using BLASTn.

RESULTS

PCR-RFLP

Although PCR-RFLP studies were carried out with a large number of restriction enzymes, data presented here represent only those that yielded applicable information for our experimental aims. PCR generated an amplicon size of 801 and 1344 bp for *CHS*2 and *PAL* exon2, respectively (Figures 1 & 2).

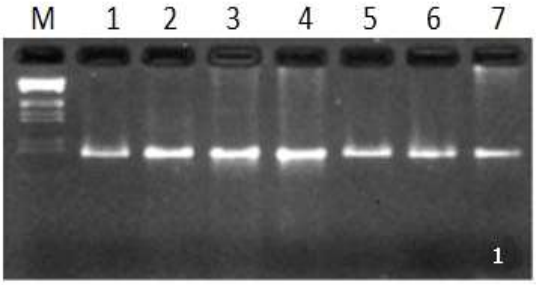

Figure 1. PCR profile of tea samples with *CHS*2 exon2 primers. *Lane M* = λ DNA/*Eco*RI + *Hin*dIII marker.

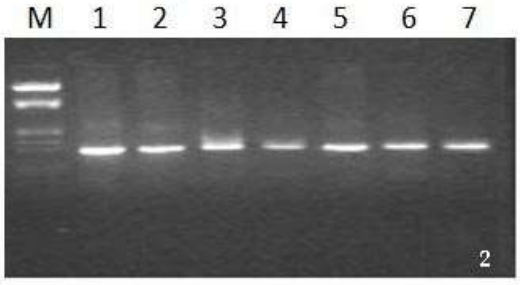

Figure 2. PCR profile of tea samples with *PAL* exon2 primers. *Lane M* = λ DNA/*Eco*RI + *Hin*dIII marker.

PCR-RFLP profile of *CHS*2 exon2 digested with *Rsa*I

Two types of profiles were observed from the restriction profiles obtained for exon2 of *CHS*2 digested by *Rsa*I (Figure 3). One profile was found to correspond to a homozygous condition for the restriction site (R1R1) and the other corresponded to a heterozygous condition for the restriction site (R1R2). We designated the homozygous condition as profile PR1 and the heterozygous condition as profile PR2. In TS463, digestion of the *CHS*2 exon2 amplicon with *Rsa*I showed only the homozygous profile PR1. In TS379, both types of profiles could be observed: parent 14.12.16 and eleven F_2 progenies showed a homozygous profile PR1, while the other parent 14.5.35 and nine progenies showed a heterozygous profile PR2. The

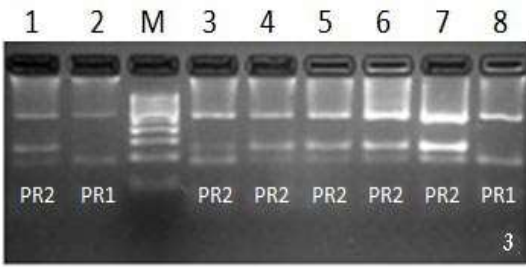

Figure 3. CAPS profile observed in the *CHS*2 exon2 amplicon digested with *Rsa*I identifying the PR1 and PR2 profiles in TS379. *Lane M* = 100-bp ladder.

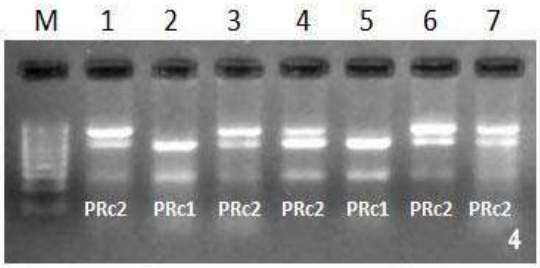

Figure 4. CAPS profile observed in the *CHS*2 exon2 amplicon digested with *Rca*I identifying the PRc1 and PRc2 profiles in TS 379. *Lane M* = 100-bp ladder.

digestion of the *CHS*2 exon2 amplicon in TS656 and TS658 showed a heterozygous profile PR2, while TS657 showed a homozygous profile PR1. This polymorphism was observed owing to two different allelic forms at this locus.

PCR-RFLP profile of *CHS*2 exon2 digested with *Rca*I

Characteristic DNA patterns obtained by digesting the *CHS*2 exon2 amplicon with *Rca*I showed two different allelic forms because only one allele contained a restriction site for this enzyme (Figure 4). The homozygote for the restriction site was assigned Rc1Rc1 and the heterozygote with the absence of a restriction site was designated Rc1Rc2. We named the profile for homozygosity at the *Rca*I restriction site as PRc1 and the profile for heterozygosity PRc2. In TS463, both parents and all twenty progenies showed a heterozygous profile PRc2. In TS379, the parent plant 14.5.35 produced two fragments while the other parent 14.12.16 revealed a heterozygous profile. Eleven progenies in TS379 showed the profile PRc1 while nine progenies showed the profile PRc2. The *CHS*2 exon2 amplicon restriction digest pattern in all three clonal seed stocks TS656, TS657, and TS658 had a heterozygous profile PRc2.

PCR-RFLP profile of *PAL* exon2 digested with *Kpn*I

A *Kpn*I restriction site in *PAL* was unevenly distributed across the samples studied, and found to be completely absent in most of the samples. In parent sample TV19 (TS463), the CAPS profile PK1of the *PAL* exon2 digested with *Kpn*I showed two restriction fragments of 385 and 959 bp (Figure 5). Parent sample TV1 (TS463) and two progenies, 10M and 17M, produced four fragments of 173, 208, 264, and 698 bp, respectively when digested with *Kpn*I. This profile was designated PK2. The rest of the progenies showed profile PK1 (Figure 6). *Kpn*I digestion of the *PAL* exon2 in samples belonging to seed stocks TS379 and TS656, and TS657 and TS658 showed two fragments consistent with the PK1 profile.

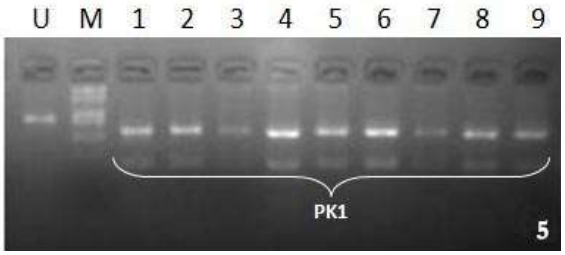

Figure 5. CAPS profile observed observed in the *PAL* exon2 amplicon digested with *Kpn*I identifying the PK1 profile in TS656. M=ë DNA/*Eco*RI+*Hin*dIII marker. U = Undigested amplicon.

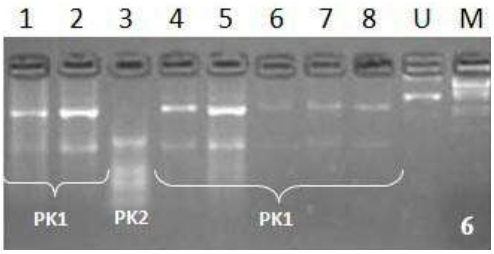

Figure 6. CAPS profile observed in the *PAL* exon2 amplicon digested with *Kpn*I with both the PKI and PK2 profiles in TS463. M = ë DNA/*Eco*RI+*Hind*III marker. U = Undigested amplicon.

Nucleotide sequence analysis

The total sequence length recovered for *CHS*2 exon2 is 760bp. After BLASTn analysis the sequence showed 98-99% homology with the *CHS*2 gene of *Camellia sinensis* cv Yabukita (NCBI Nucleotide D26596). The clustal W 2.1 sequence analysis showed that the gene is highly conserved as it is the exon region. But we have observed single nucleotide polymorphism at positions 37, 70, 232, 526 and 616 (Figure 7). At position 37, sample *C. sinensis* cv Yabukita has 'G' and all other samples have 'A'. At position 70, sample *C. sinensis* cv Yabukita has 'C' and the rest of the studied samples have 'A'. At position 232, samples 658, 658, 1M, TV1, *C. sinensis* cv Yabukita have 'G' and the rest have 'C'. At position 616, sample *C. sinensis* cv Yabukita has 'C' and the rest of the studied samples have 'T'.

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657          ACTAGATG 60   GAAGTACCA 120   AGCGCCTCA 240   GCGACGGT 540   TCCTGGT 720
1A           ACTAGATG 60   GAAGTACCA 120   AGCGCCTCA 240   GCGACGGT 540   TCCTGGT 720
15A          ACTAGATG 60   GAAGTACCA 120   AGCGCCTCA 240   GCGACGGT 540   TCCTGGT 720
14.12.16     ACTAGATG 60   GAAGTACCA 120   AGCGCCTCA 240   GCGACGGT 540   TCCTGGT 720
14.5.35      ACTAGATG 60   GAAGTACCA 120   AGCGCCTCA 240   GCGACGGT 540   TCCTGGT 720
TV19         ACTAGATG 60   GAAGTACCA 120   AGCGCCTCA 240   GCGACGGT 540   TCCTGGT 720
658          ACTAGATG 60   GAAGTACCA 120   AGCGGCTCA 240   GCGACGGT 540   TCCTGGT 720
656          ACTAGATG 60   GAAGTACCA 120   AGCGGCTCA 240   GCGACGGT 540   TCCTGGT 720
1M           ACTAGATG 60   GAAGTACCA 120   AGCGGCTCA 240   GCGACGGT 540   TCCTGGT 720
TV1          ACTAGATG 60   GAAGTACCA 120   AGCGGCTCA 240   GCGACGGT 540   TCCTGGT 720
D26594       ACTGGATG 60   GAAGTCCCA 120   AGCGGCTCA 240   GCGATGGT 540   TCCCGGT 720
             **** ***      **** ****      **** ****      **** ***       *** ***
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Figure 7. CLUSTAL W 2.1 multiple sequence alignment of *CHS*2 exon2 showing single sequence polymorphisms at the specified position. Positions 37, 70, 323, 526 and 616 respectively is represented by gaps in the asterix.

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D26596       GATCAATGTTT 300   AATACACGTC 360   AGCCGAGATCTCAGAACT 540   AGGCAGTTGAT 660   ATGCATTGA 960
6A           GATCGATGTTT 300   AATACACGTC 360   AGCCGAGATCTCAGAACT 540   AGGCAGTTGAT 660   ATGCATTGA 960
14.5.35      GATCGATGTTT 300   AATACACGTC 360   AGCCGAGATCTCAGAACT 540   AGGCAGTTGAT 660   ATGCATTGA 960
TV19         GATCGATGTTT 300   AATACACGTC 360   AGCTGAGATCTCAGAACT 540   AGGCAGTTGAT 660   ATGCGTTGA 960
12M          GATCGATGTTT 300   AATACACGTC 360   AGCTGAGATCTCAGAACT 540   AGGCAGTTGAT 660   ATGCGTTGA 960
657          GATCGATGTTT 300   AATACACGTC 360   AGCTGAGATCTCAGAACT 540   AGGCAGTTGAT 660   ATGCGTTGA 960
658          GATCGATGTTT 300   AATACACGTC 360   AGCTGAGATCTCAGAACT 540   AGGCAGTTGAT 660   ATGCGTTGA 960
656          GATCGATGTTT 300   AATACACGTC 360   AGCTGAGATCTCAGAACT 540   AGGCAGTTGAT 660   ATGCGTTGA 960
TV1          GATCGATGTTT 300   AATACACGTC 360   AGCTGAGATCTCAAAACT 540   AGGTACCTGAT 660   ATGCTTTGA 960
14.12.16     GATCGATGTTT 300   AATACACGCC 360   AGCCGAGATCTCAGAACT 540   AGGCAGTTGAT 660   ATGCATTGA 960
             **** ******       ******** *       *** *********** ****     *** *  ****      **** ****

D26596       TAGAGAGT 1080     GAACTGAATT 1140     GAAGGTCCGGTCAC 1200
6A           TAGAGAGT 1080     GAACTGAATT 1140     GAAGGTCCGGTCAC 1200
14.5.35      TAGAGAGT 1080     GAACTGAATT 1140     GAAGGTCCGGTCAC 1200
TV19         TGGAGAGT 1080     GAACTGCATT 1140     GAAGGTCCGGTCAC 1200
12M          TGGAGAGT 1080     GAACTGCATT 1140     GAAGGTCCGGTCAC 1200
657          TGGAGAGT 1080     GAACTGCATT 1140     GAAGGTCCGGTCAC 1200
658          TGGAGAGT 1080     GAACTGCATT 1140     GAAGGTCCGGTCAC 1200
656          TGGAGAGT 1080     GAACTGCATT 1140     GAAGGTCCGGTCAC 1200
TV1          TGGAGAGT 1080     GAACTGAATT 1140     GAAGGTCCGGTCAC 1200
14.12.16     TGGAGAGT 1080     GAACTGAATT 1140     GATGGTCCGATCAC 1200
             * ******          ****** ***          ** ****** ****
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Figure 8. CLUSTAL W 2.1 multiple sequence alignment of *PAL* exon2 showing single sequence polymorphisms at the specified position. Positions 238, 359,494,522,631,633, 634, 933, 1022, 1132, 1150 and 1157 respectively is represented by gaps in the asterix.

The nucleotide sequence recovered from *PAL* exon2 PCR product was 1,231bp. Computer simulations of the individual sequences were carried out with *Kpn*I and *Taq*I using the software DS GENE (version1.1) webcutter. The sequence obtained was then analysed using BLASTn which revealed that the sequences showed 98-99% homology with the *PAL* gene of *C. sinensis* cv. Yabukita. *PAL* exon2 being a conserved region of the genome showed very less polymorphism but single sequence polymorphisms were observed at positions 238, 359, 494, 522, 631, 633, 634, 933, 1022, 1132, 1150 and 1157 (Figure 8). At position 238, *C. sinensis* cv. Yabukita has 'A' and the rest of the samples have 'G'. At position 359, sample 14.12.16 has 'C' and the rest of the samples have 'T'. At 494, samples 14.12.16, *C. sinensis* cv. Yabukita, 14.5.35 have 'C' and samples TV1, 656, 657, 658, 12M, and TV19 have 'T'. At 522, sample TV1 has 'A' and the rest of the samples have 'G'. At position 631, sample TV1 has 'T' and the rest have 'C'. At position 633 sample TV1 has 'C' and the rest have 'G'. At position 634, sample TV1 has 'C' and the rest have 'A'. At position 1022 *C. sinensis* cv. Yabukita, 6A, 14.5.35 has 'A' and the rest have 'G'. At position 1150 sample have 'T' and the rest have 'A'. At position 1157, sample 14.12.16 has 'A' and the rest have 'G'.

DISCUSSION

Tea leaves are rich in plant secondary phenolics, especially flavonoids. Genetic diversity of tea in both '*assamica*' and '*sinensis*' varieties was first analyzed by Kaundun and Matsumoto (2003) using CAPS in the *PAL*, *CHS*2 and dihydroflavonol 4-reductase (*DFR*) genes. The *PAL* gene in tea is present as a single-copy gene (Matsumoto *et al*. 1994), whereas *CHS* was found to exist in three copies *CHS*1, *CHS*2 and *CHS*3 (Takeuchi *et al.* 1994). These genes are directly involved in the secondary metabolite pathway in plants leading to the biosynthesis of many secondary metabolites, including flavonoids. In the present study, the gene segments of exon2 for both *PAL* and *CHS*2 were targeted because exons constitute part of the mature mRNA transcript responsible for the synthesis of the corresponding proteins.

The existence of variation in the genomes of the different clones can be compared with the variation in catechin content (Elangbam & Misra 2016) of the different clones. Elangbam and Misra (2016) showed that the clones with heterozygous profile and homozygous profile showed a difference in the catechin content, therefore there appears to be close relation between the variation observed at the genomic level and the catechin content of the samples. The PCR-RFLP results of *CHS*2 exon2 and *PAL* exon2 suggests that variation exist in the genomes between the different clones studied. Elangbam and Misra (2016) has reported a variation in catechin content of the different clones studied and there appears to be a close relation between the variation observed at the genomic level and the catechin content of the samples. The nucleotide sequences did not show polymorphism which is significant to the catechin content. May be this is because out of the two polymorphic alleles, during the sequencing process only one of the allele got sequenced.

CAPS is a codominant marker could help us identify heterozygotes as well. Further it will also help to identify high quality Indian tea clones.

CONCLUSION

All the five tea clones taken under observation showed 98 – 99 % similarity for *CHS*2 and *PAL* genes. *PAL* gene in tea is present as a single-copy gene (Matsumoto *et al*. 1994), whereas *CHS* was found to exist in three copies *CHS1, CHS*2 and *CHS*3 (Takeuchi *et al.* 1994). Genetic variation of the targeted genes for catechin content ranges from 98 – 99 % with single nucleotide polymorphisms at some positions. Catechin content levels in the tea samples tested, ranged from 9 to 33 µg/mg (Elangbam & Misra 2016). Therefore, there exists a relation between the variation in DNA sequences and the variation in catechin content of the tea clones.

Acknowledgments

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