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EFFICACY OF SEVERAL QTL AND miRNA-SSR MARKERS TOWARDS HEALTH BENEFIT TRAITS IN AN ELITE DARJEELING TEA CULTIVAR

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ABSTRACT

Antioxidant and free radical scavenging ability of tea polyphenols are considered as health benefit traits that mainly contributed by tea catechins. Therefore understanding of genetic and molecular basis of this secondary metabolite production is of prime interest to facilitate the crop improvement program in tea plant efficiently. Some QTLs for these quality traits have already been available. In present study, 10 QTL linked SSRs and 14 newly developed microRNA based SSRs have been characterized in two Darjeeling tea cultivars having contrast characters and their progeny clone TS569 for validating possible association with catechin contents and antioxidant activities. Individual catechins and radical scavenging activity have been estimated by high performance liquid chromatography and UV-Vis spectrophotometry. Some of the polymorphic loci are indicated to be co-varied with such biochemical physiognomies. Characterization of microRNA based SSRs polymorphism are being the first time report in tea and discussed as ideal choice for further genotypic studies in tea.

Keywords: Antioxidant activity; catechins; microRNA; QTLs; SSRs; tea

INTRODUCTION

Tea is the highest consumed non-alcoholic beverage, made from young shoots of evergreen tea plant (*Camellia sinensis*). A significant share of revenue generation in major tea producing countries like China, India, Kenya, Sri Lanka by its production and export, thereby influencing the economic condition of the respective countries. In addition to its conventional refreshing ability, in recent years, the value added knowledge of tea health benefit properties have augmented the consumption of tea by health conscious people and increased the demand for quality tea marketing as well. The evidences point out the role of tea consumption in challenging the risk of neurological and cardiovascular disorder, diabetes, various types of cancer, oral disease even glaucoma too **(Hayat *et al.*, 2015; Hazra, Saha, *et al.*, 2017; Khan & Mukhtar, 2013; "Tea Advisory Panel, UK")**. This increased awareness demand the attention of scientific community for breeding new cultivars with improved yield and quality according to the choice of end users. However, unlike most of other agricultural crops, heterozygous woody perennial nature of tea plant limit the conventional breeding and improvement program in tea **(Hazra *et al.*, 2018)**. The repetitive large genome (~3GB) and polygenic inheritance nature of tea quality traits also hindered in generation of superior tea cultivar.

Generation and improvement of quality tea cultivars relies on screening the favorable strains with superior yield, quality and various abiotic and biotic stress tolerance ability **(Kottawa-Arachchi *et al.*, 2018)**. Recent trends of searching suitable biomarkers associated with desirable traits of agronomic crop is implemented in tea as well. However, the achievement in tea is still far from expectation than those of the other crops due to bottlenecks of life cycle in tea. Genome wide association studies and QTL mapping, in general, is the fundamental resource for understanding genomic background of complex phenotypic expression. Although a number of DNA markers have been reported on specific trait associated marker through mapping on different sets of breeding population **(Bali *et al.*, 2015; Kamunya *et al.*, 2010; Koech *et al.*, 2018; Ma *et al.*, 2014; Tan *et al.*, 2018)**, all of those might not be applicable for differentiation of worldwide genotypes as the genetic background may vary depending on origin and nature of segregating population **(Tan *et al.*, 2016)**. Therefore careful investigation and characterization of worldwide germplasm accessions and population is targeted to draw the roadmap towards achieving the goal of long term breeding program.

The primary value added quality underlying health benefit property of tea is mainly of its antioxidant properties and major bioactive components. Different polyphenols like catechin and flavonols which contribute up to 30% of the dry weight of the tender tea shoots (Pang et al. 2013). Among different catechin derivatives like epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), epigallocatechin gallate (EGCG), EGCG is the most abundant, up to 70% of the total tea catechins **(Saravanan *et al.*, 2005)** and these possess significant antioxidant and radical-scavenging activity **(Tounekti *et al.*, 2013; Zaveri, 2006)**. The major polyphenols like EC, EGC, ECG and EGCG are reported to have anti-arthritic effect as well. Hence, increasing the catechin content has become a major challenge for tea breeding program **(Ma *et al.*, 2014)**. Being highly abundant, polymorphic, locus specific, co-dominantly inherited and reproducible the simple sequence repeat or SSR or microsatellite markers are most popular and versatile type in plant genetic mapping studies Among the two types of microsatellite markers (genic and genomic) genic SSRs or expressed sequence derived markers are the utmost compelling for tagging and mapping of genes and QTLs **(Varshney *et al.*, 2005)**. Considering the fact, present study aim to explore and characterize some DNA marker and corresponding biochemical phenotype in order to facilitate marker trait association study in tea. One elite Darjeeling tea cultivar TS569 (released through bi-parental cross between Ambari Vegetative-2 and Tukdah-78) was considered for evaluation of the SSR markers and antioxidant trait using spectrophotometry and high performance liquid chromatography.

MATERIALS AND METHODS

Plant material and habitat

Two popular Darjeeling tea cultivars AV-2 and T-78 and a set of individual (n=10) from their pseudo-testcross progeny TS-569 of two year maturity (Figure 1A-C) were selected for the current study. Both the parents and their progeny (indicated as TS01 - TS10 hereafter) are being maintained at experimental field of Darjeeling Tea Research and Development Center, Kurseong. The study site is located between 26.8821º N, 88.2789º E with an elevation of 1530 meter above sea level and the annual average rainfall is 3235 mm. Temperature of the region varies from 8-20ºC.

Extraction of secondary metabolites

The tender 'two and a buds' of selected individuals were collected from the field immediately preserved at ice chilled condition for subsequent laboratory experimental works. Tea specific secondary metabolites extraction was performed following a modified protocol after **Liu *et al.* (2015)**. Fresh leaf tissues (500 mg) were homogenized in a mortar and pestle and extracted in 5ml of 70% methanol (v/v) with 1% formic acid (v/v). The tubes containing homogenized tissue were heated in a water bath at 80 °C for 15 min followed by centrifugation at 4 °C and 10,000 rpm for 15 min in a refrigerator centrifuge (Eppendoff 5418R). The clear supernatants were passed through a 0.45 µm membrane filter and stored at -20 °C avoiding light exposure until further analyses. The standard reference materials of catechin, epicatechin, epicatechin gallate, catechin gallate, epigallo catechin, epigallocatechin gallate and caffeine were purchased from Sigma-Aldrich, dissolved in 70% methanol as 2000 µg/ml stock concentration and stored at the previously mentioned condition. Standard reference materials were diluted from the stock when needed as working solution.

Quantification of catechin derivatives and caffeine by HPLC

High performance liquid chromatography has been carried out in a Waters Alliance MDLC Separations module and dual absorbance detector (Waters e2695-2487) equipped with a C18 reverse phase silica based column (X-Tera) with 4.6×250 mm dimension at 35°C. The double gradient elution system was standardized with mobile phase consisting of solvent A (10% acetonitrile including 0.1% trifluoroacetic acid in HPLC grade H_2O) and solvent B (90% acetonitrile with 0.1% trifluoroacetic acid). The gradient elution profile for mobile phases are as follows: 0 to 8 minute 100% A and 0% B, 8 to 14 minute 70% A and 30% B, 15 to 25 minute 100% A and 0% B at 1ml/min flow rate. The detection wavelengths have been set at 280 nm and 254 nm respectively. Different Catechin, catechin derivatives and caffeine peaks have identified (Figure 1D) and quantified based on comparing retention times and peak area of the same obtained from standard reference materials respectively. The downstream analyses have been performed using Empower Pro software from Waters ltd.

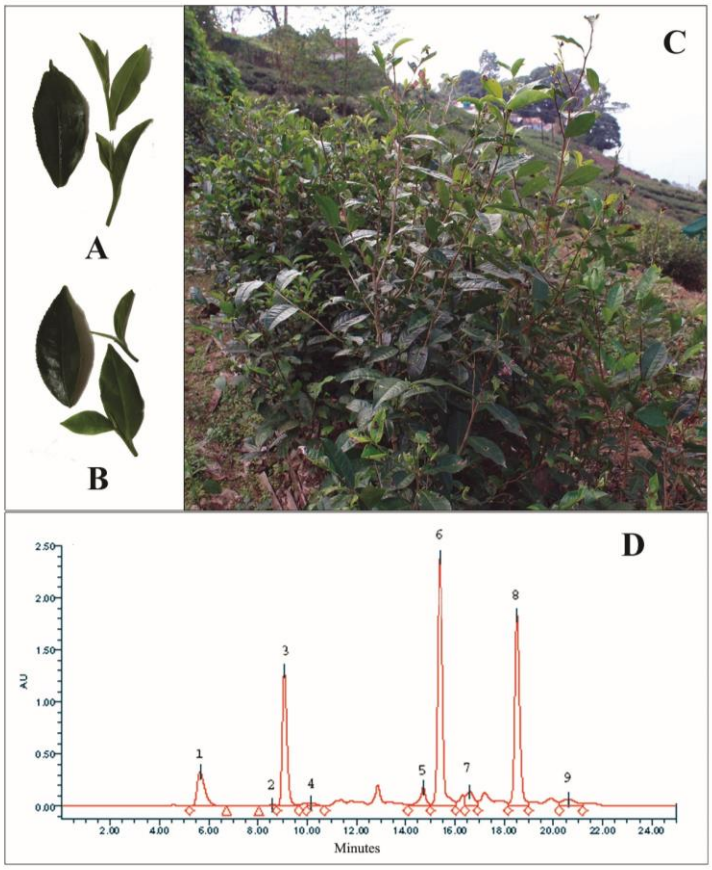

Figure 1 (A-D): Plant samples used in the study, Mature leaf and two and a bud of A – AV2, B – T78, C – Individuals of mapping population of TS569, D – Representative HPLC chromatogram with individual catechin peaks - 1 (gallocatechin; GC), 2 (epigallocatechin; EGC), 3 (caffeine), 4 (catechin; C), 5 (epicatechin; EC), 6 (epigallocatedchin gallate; EGCG), 7 (gallocatechin gallate; GCG), 8 (epicatechin gallate; ECG) and 9 (catechin gallate, CG)

Determination of free radical (ROS/RNS) scavenging activity

Superoxide radical, a reactive oxygen species scavenging activity has been measured by the nitro blue tetrazolium (NBT) reduction method **(Fontana *et al.*, 2001)**. The non-enzymatic phenazinemethosulfate (PMS) and nicotinamide adenine dinucleotide (NADH) system generates superoxide radicals, which in turn reduce (NBT) to a purple formazan. The reaction mixture consisted with 20 mM phosphate buffer (pH 7.4), 73 µM NADH, 50 µM NBT, 15 µM PMS and test sample solution. After incubation for 5 min at ambient temperature, the absorbance of generated formazanhas been measured at 562 nm of wavelength. Radical scavenging activity of the samples have been expressed by the calculated IC50 value through standard calculation method.

The nitric oxide radical, a reactive nitrogen species scavenging ability has been quantified by the Griess Illosvoy reaction **(Garratt, 2012)**. Different concentration of samples have been allowed to react with 10 mM Sodium nitropruside in phosphate buffered saline (pH 7.4). Following incubation at 25°C for 150 min, 0.5 ml of the incubated solution has been mixed with 1 ml sulfanilamide (0.33% in 20% glacial acetic acid) and allowed to stand for 5 minute. Thereafter 1 ml of napthylethylenediamine dihydrochloride (NED) (0.1% w/v) was added to it and incubated the mixture at 25°C for 30 min. The pink chromophore generated in the resultant mixture has been measured spectrophotometrically at 540nm of wavelength. Radical scavenging activity of the samples have been expressed by the calculated IC50 value through standard calculation method.

Isolation of genomic DNA

Total genomic DNA from all plant samples have been isolated using a DNeasy Plant kit (Qiagen). A total amount of 100-200 mg Fresh leaf tissues have been homogenized into fine powder with liquid nitrogen and transferred to tube containing 400 µl buffer AP1 (included in kit), 40 µl β-mercaptoethanol and 8 µl RNAse A and placed in a water bath at 65 °C for 10 minutes. The remaining steps have been followed as the manufacturer's protocol. Finally the DNA samples have been eluted in 100 µl elution buffer and stored at -20 °C until further use. Quality and quantity of the isolated DNA are being checked through agarose gel electrophoresis and a Qubit 2.0 (Invitrogen) fluorometer assay respectively.

Characterization of QTL and miRNA based SSR markers

A set of 10 different catechin QTL linked SSR markers **(Ma *et al.*, 2014)** and 14 microRNA precursor based SSR flanking primers, previously designed in this laboratory **(Hazra, Dasgupta, *et al.*, 2017)** were taken into consideration for genotyping the selected individuals of F1 progeny and their parents AV2 and T78 cultivars (Table 1). The PCR mixture contained of 25 µL total volume: 5 µl 5× flexi buffer, 0.5 mM MgCl2, 4 mM dNTPs, 10 µM of each primer, 1 U GoTaq DNA polymerase and 10 ng genomic DNA (Promega, USA). Amplification of QTL-SSRs has been performed according to the method of **(Zhao *et al.*, 2008)** using the following reaction conditions: 4 min at 94°C for initial denaturation, followed by 35 cycles of 94°C for 30s, annealing temperature for 30s, 72°C for 30 s, with a final extension at 72°C for 7 min. Amplification profile for miRNA based SSRs are being followed as: initial denaturation at 95 °C for 5 min followed by 34 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min **(Ganie & Mondal, 2015)**. The final extension was at 72 °C for 10 min. The amplicons have been separated in a 3% Agarose gel with ethidium bromide staining, visualized and photographed in a gel documentation system (Biostep, Germeny) equipped with a Olympus C7070 camera. Polymorphic bands for each primers have been manually scored and utilized the scored data for correlation analyses and interpretations.

RESULT

Catechins and caffeine contents

A wide range of catechins, EC, CG, EGC, ECG, EGCG and caffeine content were observed (Figure 2) among the sampled individuals. The most abundant of them is EGCG and the least present is CG. Out of the two parents, T78 has more amount of catechins than that of AV2. Wide variation in catechins content were observed among the individuals of F1 progeny. The F1 progeny also exhibits similar characteristics, like EGCG is the most abundant and CG to be the least. TS05 from F1 progeny has an overall higher amount of catechins. The highest amount of EGCG and caffeine is present in TS02. The catechin and caffeine content is mostly higher in F1 progeny than the parents, whereas CG, ECG, EGC, EGCG are mostly in lower amount as to the parents. Pearson's correlations between catechin compounds were calculated and appended in Table 2 It is revealed from the result that the individual catechins correlated significantly with each other, having strong positive correlation between EC and ECG (r= 0.758, P≤0.01), CG and ECG (r= 0.825, P≤0.01), CG and EGCG (r= 0.824, P≤0.01), ECG and EGCG (r= 0.869, P≤0.01).

Table 1 Primers used in the study

Type	Marker ID	Repeat motif	Forward primer sequence	Reverse primer sequence	Ta (℃)	Expected allele size (bp)
QTL-SSR	TM376	(AGATGG)3	GACGACTTGGAGGGTACGAG	CACGTATCCTTTGGCCTGTT	52	231
	TM546	(ATCTCA)3	TTGAAGTTAGCCAAGGCATC	TCCCAATCCCTTTAGTGTGC	52	115
	TM623	(CTT)6	TCCCAATCATCATCATCGAA	GGTTCCCATTGGTTTGTGTC	52	141
	TM586	(TGAGAT)4	TCGATAATACCCCCAACCAA	TCTCTCTCAAAAGTGGGCGT	52	186
	TM560	(TC)10	CAAGTTTGCGAGTCCTCCTC	GCCCACGAGGTTAGATGTGT	58	185
	TM453	(TTC)6	AAGTCACAACACCACCACCA	GAGGCAGCGATAGTACCAGG	52	268
	TM340	(ACC)9	TGAGGACGACGAAGGAT	TCCGAGCCACCGAACAT	52	113
	TM399	(TC)8	TCTCTGCTCTGCTGCTTTCA	CGATCTGGTTGGGACAAAGT	54	107
	TM412	(AG)14	GCAGGCTTCAAGGTTTTCAG	TGTTGTTCTGGCGTTGAGAG	52	215
	TM435	(CTC)6	GGTGGTGTGGTTTAGAGGGA	TTCATCTTGGGGAGCAATTC	52	242
miRNA-SSR	miSSR01	(CGC)3	GGAGTGAAGGAGGGAAGA	CCGAGAGAATAATAAGGAGGA	53	205
	miSSR02	(GAG)3	AGTGATTATTGGTGGTGGTC	TCTCAACCAATTCAACAAGTC	52	152
	miSSR03	(TA)2	CTGTTACTGCAGCTTAACCAA	AAATATGCTGCTCATTCAAAC	52	152
	miSSR04	(GGC)3	AGAAGATGTCTCAGGGAAGAG	ACACAAGTATGTCACCAGCTC	54	177
	miSSR05	(GTGGA)5	GAACCTTTCCTCCAGAATTTA	TCACATTTAGCTTTTCACTCC	50	162
	miSSR06	(GATGAC)6	ACGATGGTGGTTATGAATATG	GGGTTTTTGTTAAGTTGTTCA	50	139
	miSSR07	(TTC)3	AGACAAAAACCAAGGCTAGAT	CATCTTGTGCAGATCTCAGTT	52	149
	miSSR08	(CTC)3	TGATGATGAGGAAGGAGATAA	TTGCTTTAGTGAAACAACTCC	51	136
	miSSR09	(GA)2	TAGAACTTGCAGAGAGAATCG	TACACATTGGGAAGAAGAAGA	51	152
	miSSR10	(TTC)3	AGATACACATTGGGAAGAAGG	TAGAACTTGCAGAGAGAAACG	52	149
	miSSR11	(GGA)3	AGACACAGGCAGACATAGAGA	AAGATGCGATGAGATCAGATA	55	165
	miSSR12	(AAC)3	CGTTAGGCTATTTTGTTTCAA	TTCTGTCAATCATCCAATTTC	49	159
	miSSR13	(TC)2	TACTTCCAACCAAACACAAGT	TAGCTTACCACCTCAATCAAA	52	168
	miSSR14	(TCTA)4	CTCCTGTACACTCTCTCTCTCC	GATGAACAGCATAGGTATCCA	55	127

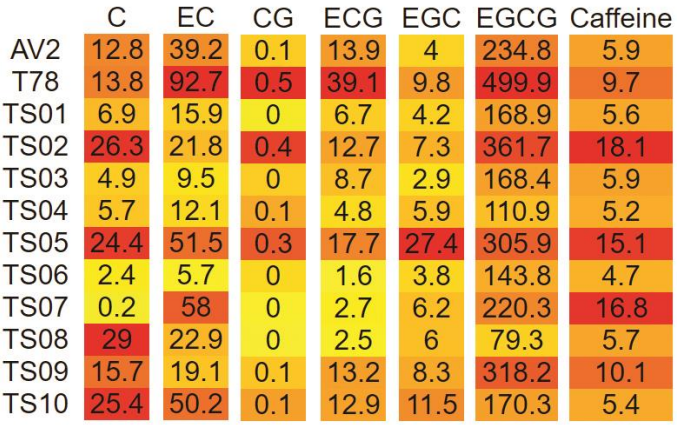

Figure 2 Concentrations of catechins and caffeine in µg/ml among studied samples

ROS/RNS scavenging activity

Free radical scavenging activity has been determined of the parents and the F1 progeny and obtained data presented by calculated IC50 values (inhibitory concentration to scavenge 50% of radicals). More IC50 value indicating to less scavenging potential or anti-oxidant capacity present in the sample. It is observed that AV2 and T78 has similar scavenging activity except T78 has slightly higher nitric oxide scavenging activity, in turn, the anti-oxidant property attribute to be low (Table 3). The progeny shows similar characteristics for superoxide scavenging efficiency as the parents whereas, nitric oxide scavenging activity is similar to that of AV2.

Table 2 Correlation among different catechin concentrations

	C	EC	CG	ECG	EGC
EC	0.184				
CG	0.402	.587*			
ECG	0.267	.758**	.825**		
EGC	0.503	0.439	0.498	0.386	
EGCG	0.197	.657*	.824**	.869**	0.357

*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).

Table 3 Antioxidant activity of studied samples

Sample	Free radical scavenging activity (IC_{50})	
	Nitric oxide	Superoxide
AV2	0.532 ± 0.008	0.322 ± 0.003
T78	0.703 ± 0.007	0.321 ± 0.003
TS01	0.498 ± 0.01	0.322 ± 0.003
TS02	0.585 ± 0.029	0.324 ± 0.003
TS03	0.524 ± 0.008	0.345 ± 0.006
TS04	0.541 ± 0.024	0.32 ± 0.006
TS05	0.516 ± 0	0.323 ± 0.004
TS06	0.589 ± 0.024	0.334 ± 0.007
TS07	0.588 ± 0.005	0.337 ± 0.002
TS08	0.583 ± 0.009	0.317 ± 0
TS09	0.535 ± 0.004	0.319 ± 0.001
TS10	0.561 ± 0.013	0.33 ± 0.001

Molecular marker based polymorphism

All the QTL-SSR markers tested in present work, produce clear and reproducible band in both the parents and their progeny genotyped at their respective loci. A total 20 alleles have been scored with 10 SSR markers among which 14 are polymorphic (70%) in nature. Maximum number of alleles (4) produced by TM560 followed by TM340 and TM399 with 3 loci each (Figure 3). In case of 14 miRNA based SSR markers, 22 loci have been scored, among which 15 are polymorphic (68%). Maximum number of loci (3 loci) reproduced by miSSR04 and most others with two of each.

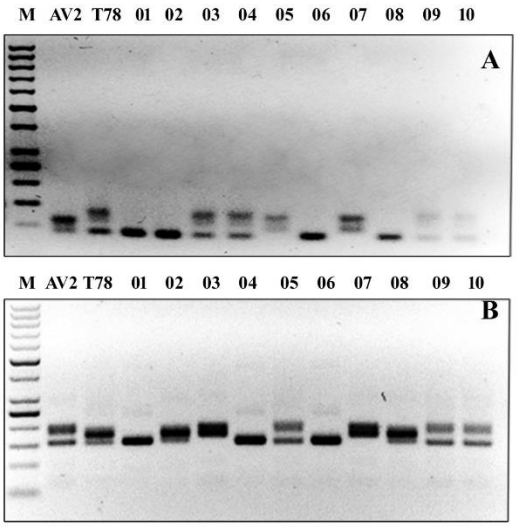

Figure 3 Representative agarose gel picture of A- QTL-SSR marker TM399 and B - miRNA-SSR-03

Marker trait correlation analysis

Point biserial correlation coefficients have been observed between different markers and biochemical traits of the tea plant (shown in Table 4). The correlation between TM399 and nitric oxide (r = 0.701, P≤0.05), TM399 and caffeine (r = -0.655, P≤0.05), TM399 and EGC(r = -0.616, P≤0.05), TM435 and nitric oxide (r= 0.685, P≤ 0.05), TM560 and superoxide (r = -0.695, P≤ 0.05). The correlations between the microRNA SSR markers and traits have also been observed. The correlations between mi01 and EC, EGCG, ECG, CG, TC are (r = -0.726, P≤0.01), (r = -0.702, P≤0.05), (r = -0.856, P≤ 0.01), (r = -0.669, ≤ 0.05), (r = -0.733, P≤ 0.01), respectively. The correlation between mi05 and EC (r = 0.600, P≤0.05), mi14 and C (r = 0.723, P≤0.01).

Table 4 Significantly correlated bands with biochemical parameters

	Marker	Nitric oxide	Superoxide	Caffeine	C	EC	EGCG	ECG	CG	EGC	TC
C06	TM399	.701*		-.655*						-.616*	
C11	TM435	.685*									
C14	TM560		-.695*								
D01	mi01					-.726**	-.702*	-.856**	-.669*		-.733**
D08	mi05					.600*					
D22	mi14				.723**						

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

DISCUSSION

Identification and QTL for agronomically desired traits and subsequent validation of marker-trait associations are key steps in developing of improved tea cultivars. As a woody perennial crop, tea agronomic traits are being attempted to link with molecular markers in order to select individuals with desired physiognomies at early stages of growth **(Koech *et al.*, 2018; Ma *et al.*, 2014; Tan *et al.*, 2016)**. Different secondary metabolites like catechin, caffeine concentration and other antioxidant traits are considered as the major contributor of tea quality in terms of its health benefit aspect. **Ma *et al.* (2014)** first identified 25 QTLs for catechins content in tea plant and reported 12 SSR markers closely linked to these QTLs. In present study 10 markers among the earlier study **(Ma *et al.*, 2014)** are cross validated in biparental mapping population of TS569 (AV2×T78). Among the tested markers, almost 70% of the resulting loci are polymorphic. However, only one of the validated markers TM399 has been found to significantly associate with EGC content among studied individuals. Though, the above marker and the other two markers (TM435 and TM560) are significantly associated with studied traits like caffeine content and radical scavenging activity. Reduced efficiency of other earlier reported markers can be attributed to the reasons, i) QTLs are critically subjective to different growing environments and several abiotic conditions during sampling time may have responsive role in altered phenotype **(Chen *et al.*, 2010; Collard *et al.*, 2005)**, ii) small sample size of studied mapping population minimal statistical support for detection of QTLs. To overcome this bottlenecks long term phenotyping with a considerably large population has to be taken into account in subsequent research. In recent times, SSR motif variation within microRNA precursor has become a marker of choice for trait association study. Some earlier studies suggested that length variations of the repeats in miRNA precursor gene interfere the stem-loop structure formation of premature miRNA and their synthesis which may lead to develop trait specific miRNA based SSR markers in plants **(Ganie & Mondal, 2015; Mondal & Ganie, 2014)**. **Mondal and Ganie (2014)** reported that SSRs within rice miRNA genes could distinguish salt tolerant and susceptible genotypes and also figured out the genetic diversity of salt sensitive genotypes. In tea, trait association study with miRNA precursor based SSR markers still unexplored. Very recently, 18 SSR motifs are found to be present in 13 predicted miRNAs from tea expressed tag sequence database and 14 primer pairs were designed from the SSR flanking region **(Hazra, Dasgupta, *et al.*, 2017)**. Current study experimentally validated all 14 miRNA based SSRs and three of them (miSSR-01, miSSR-05 and miSSR-14) has been found to be associated with certain catechin contents. Most significantly miSSR-01 has the excellent co-variability and reverse correlation with almost all catechin derivatives. Further investigation on miSSR-01points to a miR164 family precursor gene. This miR164 family members are conserved microRNAs and expressed in the leaves of tea **(Prabu & Mandal, 2010)**. **Jeyaraj *et al.* (2017)** reported that expression of *Camellia sinensis* miR164a downregulates the *NAC* transcription factor gene which controls various biotic and abiotic stress response. Another member of the family miR164b is involved in phenylpropanoid biosynthesis. Another report suggested that miR164b has potential role in regulating a catechin pathway gene anthocyanidin synthase (*ANS*) by inhibiting its translation **(Ping *et al.*, 2017)**. Therefore the repeat motif variation in the said microRNA precursor might have vital role in its formation and function which in turn influence the downstream biochemical expression and ultimately tea leaf catechin contents. As a whole, this study suggests along with reported QTL-SSR markers newly developed miRNA based SSRs can be suitable choice for trait association mapping study in tea. Moreover, highly polymorphic reproducible and regulatory non coding RNA specific SSRs are ideal for specific and sensitive genotypic application.

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