Construction of a high-density reference linkage map of tea (Camellia sinensis)

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A few linkage maps of tea have been constructed using pseudo-testcross theory based on dominant marker systems. However, dominant markers are not suitable as landmark markers across a wide range of materials. Therefore, we developed co-dominant SSR markers from genomic DNA and ESTs and constructed a reference map using these co-dominant markers as landmarks. A population of 54 F1 clones derived from reciprocal crosses between ‘Sayamakaori’ and ‘Kana-Ck17’ was used for the linkage analysis. Maps of both parents were constructed from the F1 population that was taken for BC1 population. The order of most of the dominant markers in the parental maps was consistent. We constructed a core map by merging the linkage data for markers that detected polymorphisms in both parents. The core map contains 15 linkage groups, which corresponds to the basic chromosome number of tea. The total length of the core map is 1218 cM. Here, we present the reference map as a central core map sandwiched between the parental maps for each linkage group; the combined maps contain 441 SSRs, 7 CAPS, 2 STS and 674 RAPDs. This newly constructed linkage map can be used as a basic reference linkage map of tea.

Key Words: tea, Camellia sinensis, linkage map, SSR, RAPD, STS, reference map.

Introduction

Tea (Camellia sinensis) is one of the most popular beverage crops originated in Asia, and the products are widely consumed all over the world. In 2009, the FAO estimated that 2.8 million hectares of tea fields was harvested (http://faostat.fao.org/). Harvested fresh tea leaves are processed into many types of tea, including black tea, pan-fired green tea, steamed green tea, oolong tea and powdered green tea, and the processed tea leaves are traded around the world. Some of the major tea-producing countries are developing countries, for which tea is a major source of foreign currency revenue. Tea has a genome size of 4 Gb (Tanaka et al. 2006); its basic chromosome number is n = 15 (Morinaga et al. 1929) and it is commonly diploid. Since tea is an outcrossing woody plant with a self-incompatible reproductive system (Tomo et al. 1956), its genome is always heterogeneous. Traditionally, tea trees have been propagated as seedlings, but seedling tea trees do not have synchronous bud break, uniform harvest times and uniform quality. Recently, clonal cultivars have become the main plant materials used in many countries. Clonal cultivars have synchronous bud break, uniform harvest times and uniform quality, making them well suited for machine harvesting.

To breed clonal tea cultivars, breeders have to select a large number of individuals with desirable characters from a large number of F1 segregating populations. The selected individuals are clonally propagated and screened with the information of clonal strains. After evaluation, elite clones are released as cultivars. Production of high-quality tea leaves is the most important breeding objective, but the evaluation is laborious and time-consuming. In addition to being a lengthy process, tea breeding also requires large field areas. For these reasons, it is efficient and effective to multiply early-stage populations and select breeding materials on the basis of an evaluation of DNA markers for important
agronomic characters that are controlled by a few loci, such as pest resistance, before planting the seedlings in the field. At Makurazaki Tea Research Station, a breeding system that combines marker-assisted selection (MAS) in the juvenile phase and the acceleration of generations has shown successful results (Tanaka 2006). It would be efficient to be able to perform MAS for many characters in many different populations, so a method of genetic analysis is needed for a wide range of genetic materials. It is therefore desirable to construct a reference map with many landmark markers.

A few linkage maps of tea based on the pseudo-testcross theory (Grattapaglia and Sederoff 1994) have been reported (Hackett et al. 2000, Ota and Tanaka 1999, Tanaka et al. 1995). These maps were constructed by using dominant marker systems such as Random Amplified Polymorphic DNAs (RAPDs) or Amplified Fragment Length Polymorphisms (AFLPs). By using pseudo-testcross theory, it is easy to construct accurate linkage maps for small F1 populations (AFLPs). By using pseudo-testcross theory, it is easy to construct accurate linkage maps for small F1 populations having two heterozygous parents, because the segregation ratio of each marker is 1:1 and linkage relationships among them are very simple and clear. However, the utility of dominant markers depends on the particular material being tested: they are not universal markers. In addition, the relationships among the linkage groups on the previously reported maps are unknown. In a few fruit trees, reference linkage maps have been developed by using co-dominant Cleaved Amplified Polymorphic Sequences (CAPS) and Simple Sequence Repeats (SSR) markers (Omura et al. 2000, Yamamoto et al. 2002, 2005, 2007). Here, we developed a high-density reference map of tea, using both newly developed SSR markers and some previously reported SSR and CAPS markers (Ogino et al. 2009, Taniguchi et al. 2012, Ujihara et al. 2011) as landmark markers. RAPD markers were included in the parental maps but were not used as landmark markers because of their dominant nature.

Materials and Methods

Plant materials

An F1 segregating population consisting of 54 clones was derived from reciprocal crosses between the Japanese green tea cultivar ‘Sayamakaori’ and the strain ‘Kana-Ck17’. ‘Sayamakaori’ was selected from a population derived from naturally pollinated seedlings of ‘Yabukita’ (Fuchinoue et al. 1972); the pollen parent of ‘Sayamakaori’ is believed to be germplasm introduced from China (Tanaka et al. 2001). ‘Kana-Ck17’ is germplasm introduced from China. It has been reported that Chinese tea materials have greater genetic diversity than Japanese ones (Kaundun and Matsumoto 2003, Wachira et al. 2001, Yamaguchi and Tanaka 1999). We expected there to be high heterozygosity across the genomes of these materials because of the lack of duplication in the pedigrees of the parents and because tea has an outcrossing propagation system.

Development of SSR markers

The SSR markers with names beginning with ‘TMSLA’ and ‘MSG’ (Supplemental Table 1) were developed from microsatellite-enriched libraries. These libraries, enriched for GA and GT, were developed by Genetic Identification Services Inc. (GIS, Chatsworth, CA, USA) from DNA of strain ‘KM62’, extracted by using a modified CTAB method (Tanaka et al. 2001). Sequencing was performed by using a model 310, 3100xl, or 3700 Genetic Analyzer (Applied Biosystems, Branchburg, NJ, USA). The primer sets were designed with Primer Express (Applied Biosystems) or read2Marker (Fukuoka et al. 2005) software. The SSR markers with names beginning with ‘MSE’ (Supplemental Table 1) were derived from EST databases; the corresponding primer sets were also designed with read2Marker. SSR marker TMS3 was developed by using a PCR method (Tanaka and Ikeda 2000).

SSR marker detection

DNA was extracted from fresh leaves by a method using diatomaceous earth and spin filter (Tanaka and Ikeda 2002). The PCR conditions were similar to those previously described (Tanaka et al. 2003). PCR reactions (10 μl total volume) contained 0.05 units/μl of AmpliTaq DNA Polymerase (Applied Biosystems), the attached reaction buffer, 2.0 mM MgCl2, 0.16 mM of each dNTP, 1% formamide, 2 ng/μl template DNA and 2 μl of each primer DNA. The PCR temperature conditions were based on the touchdown PCR technique (Don et al. 1991). The PCR program was as follows: 5 minutes at 94°C to completely denature the DNA; followed by 34 cycles of 30 s at 94°C, 60 s at annealing temperature (described below) and 30 s at 72°C; followed by 10 minutes at 72°C to allow complete double-strand DNA synthesis. The annealing temperature, which was 62°C in the first cycle, was reduced 0.5°C per cycle during cycles 2 to 14 and maintained at 55°C for the last 20 cycles. PCR products were post-PCR labeled (Kukita and Hayashi 2002), then separated and detected by using a model 310, 3130xl, or 3700 Genetic Analyzer (Applied Biosystems). The sizes of the amplified fragments were calculated on the basis of internal-standard DNA (Gene Scan 500 LIZ, Applied Biosystems) by using GeneMapper software (Applied Biosystems).

CAPS marker detection

Total DNA was extracted by using a modified CTAB method (Kaundun and Park 2002). PCR, restriction digestion and electrophoresis were performed according to the methods described by Ujihara et al. (2005). CAPS primer sequences are given in Supplemental Table 2.

RAPD marker detection

Template DNA for RAPD marker detection was prepared in the same way as for SSR analysis. The PCR reactions (10 μl total volume) contained 0.5 units of Ampli-Taq DNA Polymerase (Applied Biosystems), the attached reaction buffer, 2.5 mM MgCl2, 0.125 mM of each dNTP, 2 ng/μl
template DNA and 2 μM primer DNA. The PCR program was as follows: 5 minutes at 93°C to completely denature the DNA; followed by 40 cycles of 1 min at 93°C for denaturation, 1.5 min at 42°C for annealing and 1 min at 72°C for extension; followed by 10 min at 72°C to allow complete double-strand DNA synthesis. The PCR products were separated by electrophoresis in 2% agarose gel and visualized by using a UV transilluminator after being stained with ethidium bromide. The name of a RAPD marker refers to the primer name and expected band size. For example, ‘OpA_12_600’ represents the marker band of 600 bp obtained with primer OpA-12 (‘Op’ means Operon Technologies, Inc. Alameda, CA, USA).

Mapping algorithm

The F1 segregating population used in this analysis was not very large, so we formulated and applied a plan to develop a three-line reference map by using information from both parents along with the F1. This reference map was designed to provide an accurate marker orders and linkage distances between each markers, even by with limited population size. AntMap (Iwata and Ninomiya 2006) software, which is based on ant-colony optimization theory (Dorigo et al. 1996), was used to construct the linkage maps. On the basis of the double pseudo-testcross theory (Grattapaglia and Sederoff 1994), the F1 segregating population was regarded as resembling a BC1 population for the purpose of linkage analysis. Before the linkage analysis, the segregation data were sorted into data sets derived from each parent (Fig. 1). Then, a map of each parent was constructed by using the sorted data sets. In many cases, SSR marker bands generated by the same primer sets were expected to amplify the same locus in each of the two parents, so SSR markers tended to map within linkage groups in the same order in both parental maps. Thus, the amount of genotype data was doubled for those markers that detected polymorphisms within both parents. We manually selected those common markers, which were assumed to be derivatives from the same locus in both parents because of the segregation patterns of the flanking markers. This selected marker set was designated as the core marker set. We then analyzed the linkage relationships among the only core marker set on the basis of the combined (54 + 54 = 108) genotyping data and a new map was constructed that consisted of only the core marker set. This merged map was designated as the core map. To present the relationship between the linkage groups of the core map and the two parental maps, we illustrated the reference map in such a way that the core map for each linkage group was sandwiched between the parental maps by using MapChart software (Voorrips 2002).

RAPD STS

Methylene-blue-stained agarose gel blocks containing target RAPD bands were cut out and re-amplified by PCR for 15 cycles as described above for RAPD marker detection. Re-amplified PCR products were ligated into the pGEM-T vector (Promega, Madison, WI, USA). The plasmid was used to transform E. coli strain DH5α and sequenced with a 3130xl Genetic Analyzer (Applied Biosystems).

To clone the flanking sequences of RAPD STS markers, thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR, Liu et al. 1995) was performed using arbitrary primers MAT01 (CNSCTSCTNTWTCTT), MAT02 (CNCWNCANAWCAA), MAT03 (GNNGASGANAWAGAA), MAT04 (GNNGWNGTTNTWGTT) and specific primers designed by using STS sequence information. The PCR solutions (10 μl total volume) were the same as for SSR marker detection except for the arbitrary primer concentration (50 μM). The temperature program basically followed the TAIL-PCR protocol (Liu et al. 1995), integrating the touchdown PCR technique. The annealing temperature for the first PCR, which was 60°C during the first cycle, was reduced 1°C per cycle during the first 5 cycles. In the asymmetric super-cycle, the temperature for non-specific annealing was maintained at 38°C and the temperature for specific annealing started at 60°C and was reduced by 0.4°C per cycle for 15 super-cycles. For the second and third PCRs, the temperature for non-specific annealing was maintained at 38°C and the temperature for specific annealing started at 60°C and was reduced by 0.5°C per cycle for 12 super-cycles. For the third PCR, primers with single nucleotides (A, T, G or C) added to the 3’-end of the arbitrary primer sequences were used for selective amplification of the flanking sequence.

Results

Development of SSR markers

A total of 733 primer sets were designed from 1440 clones from SSR-enriched libraries and 298 primer sets were designed from 5504 tea unigene sequences obtained in our laboratory and from publicly available EST information. A total of 377 primer sets generated polymorphic amplicons between two alleles within one or both parents. The newly developed SSR markers are described in Supplemental Table 1.

Construction and characterization of linkage maps

Three-line reference linkage maps of the tea clonal cultivar ‘Sayamakaori’ and clonal strain ‘Kana-Ck17’ were constructed using SSR, RAPD and CAPS markers (Fig. 2). Details of the markers on the maps are described in Supplemental Tables 1, 2. The map of ‘Sayamakaori’ consisted of 701 loci, 363 of which are SSR markers. Similarly, the map of ‘Kana-Ck17’ consisted of 701 loci, 354 of which are SSR markers. Both maps contained 15 linkage groups, which are summarized in Table 1. All 441 SSR markers with normal segregation within the F1 population could be mapped to one of the 15 linkage groups. The maps of ‘Sayamakaori’ and ‘Kana-Ck17’ encompassed a genetic distance of 1305 cM and 1298 cM, respectively, with an average distance between markers of 1.93 cM (Sayamakaori).
Fig. 1. Pattern diagram of the construction of three-line reference map based on pseudo-testcross theory.
Fig. 2. Three-line reference genetic linkage map of tea (Camellia sinensis) using an F1 population derived from reciprocal crosses between ‘Sayamakaori’ (SAK) and ‘Kana-Ck17’ (KnCk). Locus names are given to the right of each map; genetic distances (in cM) are to the left. Core markers are denoted by bold characters.
Fig. 2. (continued)
High-density reference linkage map of tea

These two maps each included the 279 SSR markers and 2 CAPS markers in the core marker set.

The preliminary core map consisting of SSR and CAPS core markers had a large gap region in linkage group 5. To supply a marker to cover this gap, RAPD marker ‘OpT_18_200’ from the map of ‘Kana-Ck17’ was cloned and sequenced. Because there is no polymorphism for this sequence in ‘Sayamakaori’, the flanking sequence was amplified by using TAIL-PCR and sequenced. There were 2 SNPs revealing heterozygosity in ‘Sayamakaori’ in the flanking sequence of ‘OpT_18_200’ (accession numbers: AB623060 [Sayamakaori], AB623061 [Kana-Ck17]); these SNPs were mapped to the same locus as RAPD marker ‘OpM_11_750’ on the ‘Sayamakaori’ map. The segregation patterns obtained for ‘OpM_11_750’ and the new marker were identical, so we mapped the new STS marker (designated ‘OpM_18_200STS’) onto the core map.
The revised core map contained 15 linkage groups with a total length of 1218 cM. The average distance between markers was 4.35 cM, without any gap region.

**Discussion**

*Characterization of the newly constructed linkage maps*

The core map and the parental maps each covered 15 linkage groups; this corresponds to the basic chromosome number \( n = 15 \) of tea. All 441 SSR markers with normal segregation within this population could be mapped to one of the 15 linkage groups. The total length of the core map is 1218 cM, and that of the combined (three-line) map is 1317 cM. The genome size of tea is estimated to be 4 Gbp (Tanaka et al. 2006), so 10 cM of genetic distance would correspond to 30 Mbp of physical distance on average.

*Illustration and application of the reference linkage map*

We have illustrated the reference map in such a way that the core map for each linkage group is sandwiched between the corresponding parental maps. Although obtained from the same F1 population, the sets of linkage information for each parent are independent. If the maps of the two parents were to be merged into a single map by using the landmark markers, the order of the non-landmark markers would be expected to be very inaccurate. This three-line manner of illustration presents reasonably accurate marker orders.

It is common to have inaccurate marker orders in linkage
Table 1. Summarized data of each linkage group of tea (*Camellia sinensis*) reference map

| Linkage group | Genetic distance (cM) | Total distance (cM)* | Number of loci | SSR loci | RAPD loci | CAPS and STS loci |
|---------------|-----------------------|----------------------|----------------|----------|-----------|------------------|
| Core          | 110                   | 120                  | 18             | 18       | 18        | 0                |
| Sayamakaori   | 120                   | 43                   | 21             | 22       | 22        | 0                |
| Kana-Ck17     | 108                   | 44                   | 27             | 17       | 17        | 0                |
| Linkage group 2 | Core                | 104                  | 106            | 17       | 17        | 0                |
| Sayamakaori   | 102                   | 32                   | 17             | 15       | 15        | 0                |
| Kana-Ck17     | 106                   | 40                   | 22             | 18       | 18        | 0                |
| Linkage group 3 | Core                | 97                   | 100            | 23       | 23        | 0                |
| Sayamakaori   | 101                   | 62                   | 31             | 28       | 28        | 3                |
| Kana-Ck17     | 111                   | 60                   | 32             | 28       | 28        | 0                |
| Linkage group 4 | Core                | 98                   | 98             | 21       | 21        | 0                |
| Sayamakaori   | 108                   | 52                   | 26             | 25       | 25        | 1                |
| Kana-Ck17     | 72                    | 55                   | 28             | 27       | 27        | 0                |
| Linkage group 5 | Core                | 67                   | 97             | 13       | 12        | 1                |
| Sayamakaori   | 94                    | 37                   | 15             | 21       | 21        | 1                |
| Kana-Ck17     | 86                    | 38                   | 16             | 26       | 26        | 1                |
| Linkage group 6 | Core                | 86                   | 92             | 31       | 31        | 0                |
| Sayamakaori   | 92                    | 85                   | 45             | 40       | 40        | 0                |
| Kana-Ck17     | 88                    | 75                   | 34             | 41       | 41        | 0                |
| Linkage group 7 | Core                | 84                   | 91             | 14       | 14        | 0                |
| Sayamakaori   | 92                    | 24                   | 16             | 8        | 8         | 0                |
| Kana-Ck17     | 86                    | 31                   | 19             | 12       | 12        | 0                |
| Linkage group 8 | Core                | 90                   | 90             | 31       | 31        | 0                |
| Sayamakaori   | 96                    | 69                   | 41             | 27       | 27        | 1                |
| Kana-Ck17     | 97                    | 59                   | 39             | 19       | 19        | 1                |
| Linkage group 9 | Core                | 85                   | 87             | 23       | 23        | 0                |
| Sayamakaori   | 71                    | 52                   | 32             | 19       | 19        | 1                |
| Kana-Ck17     | 97                    | 60                   | 30             | 30       | 30        | 0                |
| Linkage group 10 | Core               | 78                   | 84             | 16       | 16        | 0                |
| Sayamakaori   | 86                    | 43                   | 21             | 22       | 22        | 0                |
| Kana-Ck17     | 90                    | 45                   | 19             | 26       | 26        | 0                |
| Linkage group 11 | Core               | 74                   | 79             | 15       | 15        | 0                |
| Sayamakaori   | 77                    | 29                   | 19             | 10       | 10        | 0                |
| Kana-Ck17     | 77                    | 35                   | 22             | 13       | 13        | 0                |
| Linkage group 12 | Core               | 60                   | 75             | 16       | 16        | 0                |
| Sayamakaori   | 58                    | 39                   | 23             | 16       | 16        | 0                |
| Kana-Ck17     | 80                    | 48                   | 21             | 26       | 26        | 1                |
| Linkage group 13 | Core               | 62                   | 73             | 15       | 15        | 0                |
| Sayamakaori   | 65                    | 47                   | 21             | 26       | 26        | 1                |
| Kana-Ck17     | 70                    | 38                   | 16             | 22       | 22        | 0                |
| Linkage group 14 | Core               | 62                   | 64             | 20       | 20        | 0                |
| Sayamakaori   | 85                    | 62                   | 28             | 34       | 34        | 0                |
| Kana-Ck17     | 58                    | 47                   | 22             | 25       | 25        | 0                |
| Linkage group 15 | Core               | 61                   | 61             | 6        | 6         | 1                |
| Sayamakaori   | 57                    | 25                   | 7              | 17       | 17        | 1                |
| Kana-Ck17     | 73                    | 26                   | 7              | 18       | 18        | 1                |
| Total         | Core                 | 1218                 | 1317           | 279      | 276       | 0                |
| Sayamakaori   | 1305                  | 701                  | 363            | 330      | 330       | 8                |
| Kana-Ck17     | 1298                  | 701                  | 354            | 348      | 348       | 4                |

Total marker number without duplication: 1123 441 678 9

*a* Total distance is calculated by Core linkage group plus extra region of parent maps.

*b* The number of RAPD loci does not include RAPD-STS.
maps because of missing data, especially when the population size is small. In this study, we used automated DNA sequencers to detect the sizes of amplified fragments from the SSR marker analysis. In this method, some background peaks hampered the detection of particular alleles; for example, this occurred with MSG0213 in linkage group 4 of ‘Sayamakaori’ and MSE0230a in linkage group 3 of ‘Kana-Ck17’. For such markers, the core map should be more accurate than either parental map because it is based on double the amount of linkage data per marker.

In addition, the core markers are likely to be more informative and more useful than other markers for genetic analysis in other populations. For a marker to be designated as a core marker, both parental materials had to be heterozygous for the marker and the segregation of the alleles derived from one parent had to be detectable without the interference of alleles from the other parent. That is, if the genotype of one parent was AB, the genotype of the other parent had to have been AC, BC or CD. In other words, a core marker must have had at least three distinguishable alleles within the two parental lines. To select markers for genetic analysis, it is efficient to choose markers firstly from the core marker set, secondly co-dominant markers on the parental maps. If there are no polymorphic markers within the target area, it is possible to search SNP markers from the sequence information of SSR markers. Even if no SSR markers are found in the target region, it is possible to search SNPs from the sequence of flanking region of SSR markers, or even from the sequence of an RAPD band or its flanking region, as was done here in the case of ‘OpT_18_200’ STS.

The linkage map presented here should be useful as a reference map of tea. This reference map will play an important role in setting the alignment of genomic sequences of tea as we work toward full-genome sequencing. In the future, we expect that many additional markers will be developed and mapped and these will further advance the accuracy and density of the available maps.

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