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Functional characterization of caffeine synthase genes from wild coffee, and discussion of those molecular evolutions.

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Introduction: *Coffea* genus, including three cultivated coffee species (*Coffea arabica*, *C. canephora*, and *C. liberica*), have different caffeine concentrations respectively [1]. Examining those caffeine biosynthetic enzymes and exploring its molecular evolution, we reveal how has *Coffea* genus obtained and developed caffeine synthetic ability.

Methods: Total RNA was extracted from young leaves of wild coffee species, and then cDNAs were synthesized. PCR was performed with those cDNAs as templates and oligonucleotides synthesized from the sequence information of caffeine synthetic enzymes from *C. arabica* as primers. Isolated genes were compared with corresponding *C. arabica* genes and classified into each other. Subsequently, recombinant enzymes for the identified genes were obtained using the *E. coli* expression system. To determine the substrate specificity, those recombinant enzymes were used to react with xanthine derivatives that are a caffeine precursor and nicotinic acid as substrates, and then the products were detected by TLC.

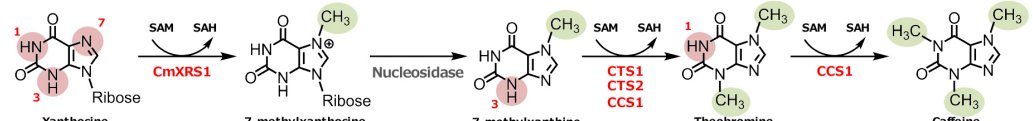

Fig.1. Biosynthetic pathway of caffeine in coffee.
Caffeine is synthesized from xanthosine via a pathway that has three SAM-dependent methylation steps. Abbreviations: CmXRS, coffee 7-methylxanthosine synthase; CTS, coffee theobromine synthase; CCS, coffee caffeine synthase; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosyl-L-methionine.

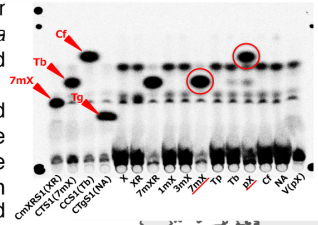

Fig.2. TLC assay of Cps-TS1.
Cps-TS1 was isolated from *C. pseudozanguebariae*. Cps-TS1 can catalyze 3*N*-methylation of 7-methylxanthine but not 1*N*-methylation. Though Cps-TS1 had highly homologous to CCS1 (90% identity), the activity was the same as CTS series (84% identity).

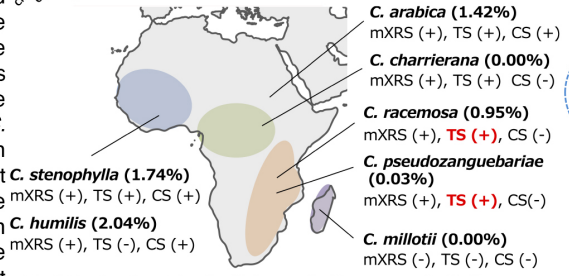

Fig.4. Regional variety of caffeine synthetic enzymes in *Coffea* genus.
The number of the right side of species is caffeine content(%). Cluster II' type TS is indicated in red letter. (+) means active. (-) means that the corresponding genes were not isolated. CS, TS, and mXRS indicate the same as Fig.1. The genus *Coffea* reseeding to East Africa has a specific TS enzyme belonging to cluster II'. Although *C. racemosa* and *C. pseudozanguebariae* have low caffeine content, it is thought that they contain theobromine.

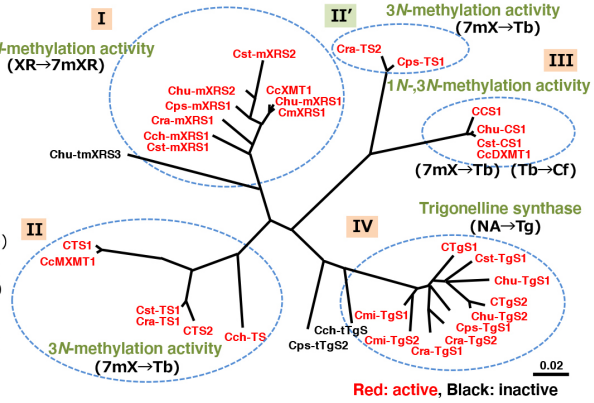

Fig.3. Phylogenetic tree of caffeine synthase family in *Coffea* genus.
Active and inactive recombinant enzymes are indicated in respectively red and black letters. Clusters I, II, III, and IV correspond to 7-methylxantosine synthases, theobromine synthases, caffeine synthases, and trigonelline synthases, respectively. Cluster II' correspond to novel type theobromine synthases because those are highly homologous to caffeine synthases (Cluster I) but have theobromine synthases activity (Cluster II). Abbreviations: CcDXMT, *C. canephora* dimethylxanthine methyltransferase; CcMXMT, *C. canephora* monomethylxanthine methyltransferase; CcXMT, *C. canephora* xanthosine methyltransferase; Cch-, *C. charrierana*; Chu-, *C. humilis*; Cmi-, *C. millotii*; Cps-, *C. pseudozanguebariae*; Cra-, *C. racemosa*; Cst-, *C. stenophylla*; TgS, trigonelline synthase; CS, TS, and mXRS indicate the same as Fig.1.

Results: We isolated five, two, five, six and four caffeine synthetic genes from *C. stenophylla*, *C. millotii*, *C. racemosa*, *C. humilis*, and *C. pseudozanguebariae*, respectively, that are highly homologous to those corresponding genes from *C. arabica*. Each of these genes had a full length of approximately 1200 bp and had the homology with the corresponding genes from *C. arabica* was over 94% as a nucleotide level. However, since the enzyme gene corresponding to *CmXRS*, *CTS* and *CCS* were not isolated from *C. millotii*, it was considered that the low or no caffeine content species had extremely low or no expression of those genes. Furthermore, when a molecular phylogenetic tree covering the caffeine biosynthetic genes from the *Coffea* genus was constructed, it was classified into four clades depending on the substrate specificity.

Conclusion: Recombinant enzymes of the genes with high homology to CCS isolated from *C. racemosa* and *C. pseudozanguebariae* did not have the same activity as CCS but only as CTS activity. It was revealed that there is a novel caffeine synthase gene class, "highly homologous to CCS but the activity is CTS." The genus *Coffea*, which originated in East Africa, may have obtained and evolved its caffeine synthetic ability as it expanded to West Africa.

References: [1] Hamon, P. *et al.*, *Coffee in health and disease prevention*, Chapter 5, p39-44 (2015) Academic press. [2] Hamon, P. *et al.*, *Mol. Phylogen. Evo.* 109, p351-361 (2017).