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Characterization of Lupeol, Linalool, and Squalene Synthase Expression Within Floral, Leaf, and Seed Tissues of *Camellia japonica* and *Camellia sasanqua*

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Abstract

Camellia *japonica* (tsubaki) and Camellia *sasanqua* (sazanka) seed oils are both marketed as tsubaki oil, a traditional Japanese beauty product applied to the hair and face. However, differences in cosmeceutical benefits between C. *sasanqua* and C. *japonica* oils are not yet fully understood. Here we aim to characterize differences in expression of antioxidant-synthesizing enzymes between the *sasanqua* and tsubaki species of Camellia. Using semi-quantitative RT-PCR, we compared gene expression of linalool synthase, squalene synthase, and lupeol synthase from Camellia seeds, petals, and leaves. C. *japonica* had a greater expression of linalool synthase in seeds relative to C. *sasanqua*. Within C. *japonica* , linalool expression was greater in leaves than in seeds. Due to the challenges of degenerate primer design, primer efficiency varied for each of the three genes studied. Nevertheless, the results suggest there is a difference in expression, and possibly a difference in antioxidant profiles, between the two species commonly used for tsubaki oil. Better understanding the concentrations of secondary metabolites between species and tissues could lead to improving the quality and efficiency of *C. japonica* and *C. sasanqua* oils as cosmeceuticals.

Introduction

Camellia japonica oil, or tsubaki oil（椿油）in Japanese, is the natural oil made by cold-pressing the seeds of a C. *japonica* plant, has been used in Japan and other East Asian countries since ancient times. It saw a particular rise in popularity during the Edo period (1603-1868) when it became commonly used for hair grooming. It is often used as a moisturizer for skin and hair because of it's sebum like composition (Jung et al., 2007), which allows it to penetrate multiple layers of skin while retaining moisture. Tsubaki oil is also noted to have a high content of oleic acid, a fatty acid that stimulates hair growth and has anti-inflammatory properties (Keis et al., 2007). While tsubaki oil popularity has died down a little since the Edo period, there are still a few big companies that continue to produce the oil, such as Oshima Tsubaki and Kaneda. One thing to note about tsubaki oil however, is that while tsubaki oil bears the same name as the *C. japonica* variety of camellias, the oil of another variety, *Camellia sasanqua*, simply sazanka in Japanese, is also distributed as tsubaki oil. Tsubaki and sasanqua plants look very similar, and are usually distinguished by *sasanqua*'s smaller flower and leaf size. *Sasanqua* petals also tend to fall off one by one, where tsubaki petals come off all at once. With regards to the growth of the plants, *sasanqua* tend to grow faster (Moor, 2020) and in cooler climates and tsubakis tend to grow to a taller height. It is believed that

*sasanqua*s were originally grown for practical purposes, but by the 14th century, the decorative cultivars were prized, similar to tsubaki (Kennedy, 2016). Other than physical differences between the plants, there is not much that distinguishes tsubaki and *sasanqua* immediately.

Most secondary metabolites (SM) are derivatives of primary metabolites and are no longer essential to the plant's survival. SMs are key factors when it comes to producing perfume, agrochemicals, and cosmetics. The amount of secondary metabolites produced depends on the area of the plant (root/stems, leaves, flowers, fruits), the age of the plant, and the period of the plant's life cycle at the moment the sample is taken. The color and scent performance of a plant may be affected by secondary metabolites, such as flavonoids, terpenoids, and other volatives can attract or repel insects and herbivores, "while toxins can be involved in plant-plant allelopathic interactions" (Hadacek, 2002).

C. japonica leaves have been found to produce antioxidant, anti-inflammatory (Kim et al. 2012) and anticancer medicinal compounds such as lupeol, squalene, and linalool (Majumder et al, 2020). Lupeol, most highly expressed in the leaf, is an anti-inflammatory and anti-microbial triterpene that is derived from oxidosqualene. It is perhaps the most studied of the three terpenes in conjunction with *C. japonica* because of its significance to the study of cosmetic tsubaki oil. Lupeol has been found to alleviate the toxicity induced by benzoyl peroxide, a chemical widely used in skin care products containing tsubaki oil (Saleem et al., 2001).

Linalool, on the other hand, a terpene alcohol found in flowers and plants and synthesized by linalool synthase enzymes, is perhaps more well known for being found in lavender than tsubaki. It gives off floral aromas that are often used in commercial products (Johnson, 2017). The antioxidant effects of linalool are most noticeable when absorbed by the bloodstream where it flows to the central nervous system "resulting in observed changes in mood and physiology." Squalene, synthesized by squalene synthase enzymes, is a triterpene that is an "intermediate in the cholesterol biosynthesis pathway." Not much about squalene in relation to C. *japonica* has been studied, but the structure of squalene is nonpolar which makes it particularly compatible with oils such as tsubaki oil. A highly efficient emollient, squalene oils quickly penetrate deep skin, locking in moisture and acting as "oxygen-scavenging agent[s]" which makes it an ideal antioxidant (Huang et al., 2009). There are many papers explaining the properties of lupeol, linalool, and squalene; what is not known is if there are any differences in antioxidant properties between sasanqua and tsubaki species of *Camellia*. Here, we aim to identify any significant differences in expression of antioxidant synthesizing enzymes between ***sasanqua*** and tsubaki species of *Camellia*.

Procedure

Sample Collection

Leaves, seeds, and petals were collected from three *C. japonica* and four *C. sasanqua* plants from primarily the Aoyama Cemetery area.

CJ1, Itakura Food Store Hiroo, 2/9/20. CJ2, Aoyama Cemetery Nishiazabu, 2.9.20. CJ3, Aoyama Cemetery Minami Aoyama, 2/9/20. SJ1, Aoyama Cemetery Office, 2/9/20. SJ2, Aoyama Cemetery Office, 2/9/20. SJ3, Aoyama Cemetery Nishiazabu, 2/9/20. SJ4, Aoyama Cemetery Office, 2/9/20. (Figure 1 Map and legend of sample collection location)

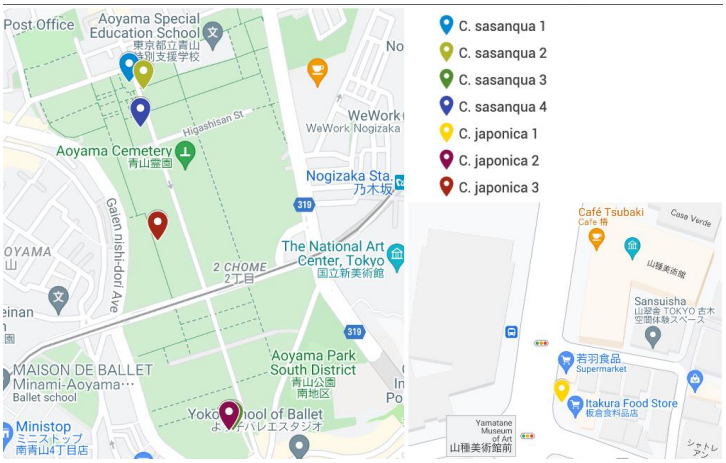

FIGURE 1: Map and legend of C. japonica and C. sasanqua tissue sample collection locations.

TABLE 1: Primer Designs indicating forward and reverse primers for genes Squalene 1, Squalene 2, Lupeol 1, Lupeol 2, Linalool 1, Linalool 2, and housekeeping gene 18S from Li et al. (2016).

Gene	Forward Primer	Reverse Primer	Sequence ID	Amplicon size
Squalene 1	TTTCTCGCAGTTTCGCCC	AAAATGCCAGTCACGGTCA	MT151371.1 (*C. vietnamensis*)	185
Squalene 2	GTCAAAGCTGTGGAATGCC	TTAGCAGTAAGACCACGCC	MT151371.1 (*C. vietnamensis*)	212
Lupeol 1	CACATAGAAGGGCACAGCAT	ATCGAAAGCCAAGTCTTCCC	XM_028206589.1 (*C. sinensis*)	176
Lupeol 2	CGGTTGGCAAGTCTCAGATT	AAGACCCTGCTTTTACTGGC	XM_028245768.1 (*C. sinensis*)	188
Linalool 1	TAACGCCGATTCTCTTTGGG	TTCCAAACCCCATGTCACTG	XM_028239882.1 (*C. sinensis*)	182
Linalool 2	CAGTGACATGGGGTTTGGAA	CACCTTTGAACTGCCTCTGT	XM_028239882.1 (*C. sinensis*)	198
18S (Li et al, 2016)	TCTCAACCATAAACGATGCCGACCAG	TTTCAGCCTTGCGACCATACTCCC	U42851.1 (*C. japonica*)	119

Primer Design

We looked for primer designs in the papers referenced but none had a design that was available and matched what we were looking for—squalene, linalool, and lupeol in *C. japonica* and *C. sasanqua*. We then used NCBI BLAST to locate nucleotides with similar sequences to *C. japonica* and *C. sasanqua* and found Camellia sinensis and Camellia vietnamensis.

Through ClustalO validated conservation, we ended up using *C. vietnamensis* for squalene as it was the most conserved. It also led us to believe we could use *C. sinensis* as a template when no other sequences were available to use for linalool and lupeol in *C. japonica* and *C. sasanqua*.

RNA Isolation from Seedlings

C. *japonica* and C. *sasanqua* RNA were isolated using the RNA Plant and Fungi isolation kit (Takara Bio Cat. #: U0949B) and the RNA Plant Isolation Kit from Machery Nagel (Cat. #: Takara U0949S) as instructed with a few minor modifications. Samples CJ-P3, CJ-L3, CS-P2, CS-L2, CS-P1, CS-L1 were extracted using the Plant Isolation Kit, while the remaining samples were isolated using the Plant and Fungi Isolation Kit. *C. japonica* and *C. sasanqua* leaf, seed (without the shell), and petal tissue was homogenized using a mortar and pestle without liquid nitrogen. 20 mM Isopropanol was used for precipitating RNA in place of ethanol.

RNA purity and concentration was determined using a UV-Vis Spectrophotometer (Model #: ASUV-1110). Samples CS-S4 and CS-L3 were slightly yellow. A possible reason for the low 260/280 ratio, or purity, is that the samples were not fully processed until two weeks after being placed in the lysis buffer (Figure 2) and frozen at -20°C. Table 2.

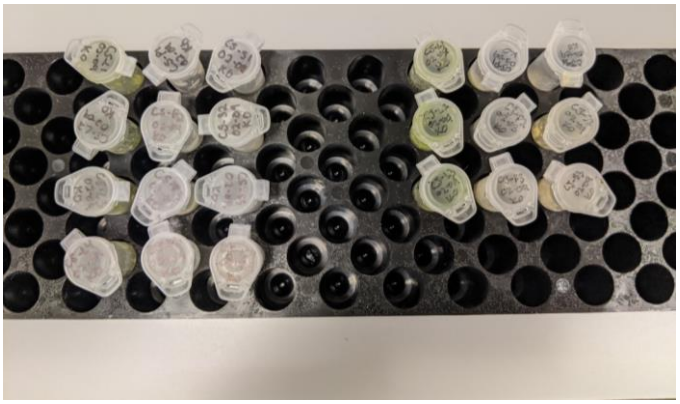

FIGURE 2: C. japonica, C. sasanqua seed, leaf, and petal tissue during RNA isolation.

cDNA Synthesis

The 1st strand of cDNA was synthesized using the PrimeScript 1st Strand cDNA Synthesis Kit as described (Cat. # 6110A). No reverse-transcriptase (no-RT) controls were also created for each biological sample.

Semi-quantitative RT-PCR

Sample CJ-L2 was used to make the amplification curve for each primer set. The sample was tested at 22, 24, 26, and 28 cycle lengths. 28 cycles were determined to be optimal for analysis. PCR were conducted as follows: 25 uL of 2X EmeraldAmp MAX PCR Master Mix (Cat. #RR320A), 1 uL of cDNA, 1uL (2 uM) each of forward and reverse primers, and 22 uL of ddH2O. All PCR conditions were as follows: 98℃ for 2 minutes; 98℃ for 15 seconds, 60℃ for 30 seconds, and 72℃ 1 minute, repeat for 28 cycles. PCR reactions were subsequently run on a 1% agarose gel using the blueGel system from MiniPCR. To quantify band size, RAW photos were taken and analyzed using FIJI, or ImageJ Bio-Formats plug-in (Schindelin et al., 2012).

TABLE 2: Purity and Concentration of C. japonica, C. sasanqua leaf, petal, and seed tissue RNA. "CS" refers to C. sasanqua samples and "CJ" to C. japonica. "L" indicates leaf tissue, "P" indicates petal tissue, and "S" indicates seed tissue.

Sample	Ratio (260/280)	A260	Concentration (ng/µL)
CS-L1	.5	.004	16
CS-L2	1.21	.017	27.2
CJ-P1	.86	.033	52.8
CS-P2	1.375	.011	17.6
CS-S2	1.32	.037	59.2
CS-S3	.71	.103	186.8
CS-S4	1.03	0.198	316.8
CS-P1	1.14	0.016	25.6
CS-P3	0.94	0.050	80
CS-P4	1.13	0.148	236.8
CS-L3	0.96	0.105	168
CS-L4	1.11	0.113	180.8
CJ-P2	0.96	0.027	43.2
CJ-P3	1.1	0.011	17.6
CJ-L1	1.16	0.029	46.4
CJ-L2	1.22	0.084	134.4
CJ-S1	1.17	0.041	65.6
CJ-S2	1.15	0.031	49.6
CJ-S3	1.5	0.081	129.6

Results

Our study found that, between *C. japonica* seeds and *C. sasanqua* seeds, C. *japonica* had a higher expression of linalool than *C. sasanqua* (Table 3).

TABLE 3: Gene expression of linalool, using the Lin1 primer set, in samples CJ-S2 and CS-S4. "CS" refers to C. sasanqua samples and "CJ" to C. japonica. "S" indicates seed tissue. "HKG" is housekeeping gene 18S.

Sample	True Gene Expression (% of HKG)
CJ-S2	76.1
CS-S4	51.1

Additionally, as shown in Table 4, linalool expression was also found to be higher in *C. japonica* leaves for both primers tested than in *C. japonica* seeds. Between linalool and squalene found in C. *japonica*, squalene had higher expression (Table 4).

TABLE 4: Expression of linalool and squalene in C. japonica leaves.

Primer	True Gene Expression (% of HKG)
Linalool 1	129.5
Linalool 2	42.2
Squalene 2	148.5

Further studies with more biological and technical replicates are advised as genomic DNA contamination may have occurred, as suggested by linalool cDNA being lower than linalool no-RT in CS-S2. It is also to be noted that primers were chosen using a degenerate primer design due to a lack of known sequences, and consequently the Linalool 1 and 2 primers ended up with a difference in primer efficiency and therefore a difference in expression levels. Though the preliminary data suggests differences between C. *japonica* and C. *sasanqua* linalool expression, further studies are needed to determine whether these differences are significant or not.

Conclusions

The secondary metabolites linalool, lupeol, and squalene synthases are what create linalool, lupeol, and squalene in the plants, specifically to this experiment, C. *japonica* and C. *sasanqua*. Tsubaki oil, or oil from the seeds of C. *japonica* and C. *sasanqua*, has been used cosmetically for the hair and face in Japan since ancient times, and for good reason: the three terpenes together contain antioxidant and anti-inflammatory properties which can promote the growth of hair by maintaining moisture in the scalp and follicles. Further research about the presence and concentration of secondary metabolites in various tissues of C. *japonica* and C. *sasanqua* will no doubt help our understanding of the differences between the two species, and this in turn will help tsubaki oil producers understand what to use in order to produce the best quality tsubaki oil possible.

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