様式 Form 7
（外国人招へい研究者）

2024 年　02　月　21　日

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独立行政法人日本学術振興会理事長　殿
To: President, Japan Society for the Promotion of Science

研究活動報告書
Research Report

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受入研究機関・部局・職 Name of Host Institution, Department and Title	国立研究開発法人産業技術総合研究所・生物プロセス研究部門・研究グループ長
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３．採用期間/ Fellowship Period

2023 年　03　月　01　日　～　2023 年　12　月　31　日

４．研究課題/ Research Theme

Understanding the terpenoid biosynthesis in *Cleome arabica* and its DsRNA-mediated regulation

５．研究活動報告/ Research Report

（１）研究活動の概要・成果/ Summary of Research Results

This project focused on studying the biosynthetic pathway of terpenoids in *Cleome arabica*. This plant is an appreciated species of Capparidaceae in North Africa for its medicinal and biological attributes (Ladhari et al., 2013, Afr.J.Bot. 88, 341-351). This plant produces a wide range of active molecules mainly specific dammarane type triterpene that are involved in fundamental physiological and ecological processes. However, the isolation and purification of the active compound from this medicinal plant involves long and complicated procedures, and expensive or impossible to synthesize chemically. Due to the low amounts of this active compound in naturally growing plant, it becomes important to increase their yield in plant or to find an alternative source of this active compound.

The aim of this study is to understand the enzymes involved in the biosynthetic pathway of dammarane type triterpene as new active compound in *C. arabica*. On the other side, we focused to establish a system to shift the metabolic pathway of plant through the direct exogenous application of dsRNA. The direct application of dsRNA is considered as a relatively rapid and low-cost method compared to plant transformation; therefore, this approach is selected here to study the effect of silencing the candidate enzymes involved in the regulation and biosynthetic pathway of triterpene in *C. arabica*. This strategy will be implicated to shift the metabolic pathway and to suppress the expression of competitive

metabolic pathways in order to enhance the production of terpenoids in *C. arabica*.

Identification of genes involved in biosynthetic pathway of terpenoids in Cleome arabica

In the first objective we focused to find candidates of the enzymes involved in biosynthetic pathway of terpenoids in *C. arabica* (Fig. 1). In fact, the molecular basis of the regulation of terpenoids synthesis in *C. arabica* is still not thoroughly understood. This study has been initiated with RNA extraction from different plant tissues of *C. arabica* for transcriptomic analysis in order to identify the gene involved in the biosynthetic pathway of terpenoids in *Cleome arabica* (Fig. 2). The Illumina Hiseq™ 2000 platform showed in total, 156,700,728 bp clean reads were generated with 43.85% GC after filtering out adaptor sequences, ambiguous reads. All clean reads were assembled into 71,907 unigenes with a total length of 96,791,710 bp. The unigene contig N50 was 1,478 bp. The size of unigenes ranged from 201 bp to 17,024 bp with an average length of 584 bp.

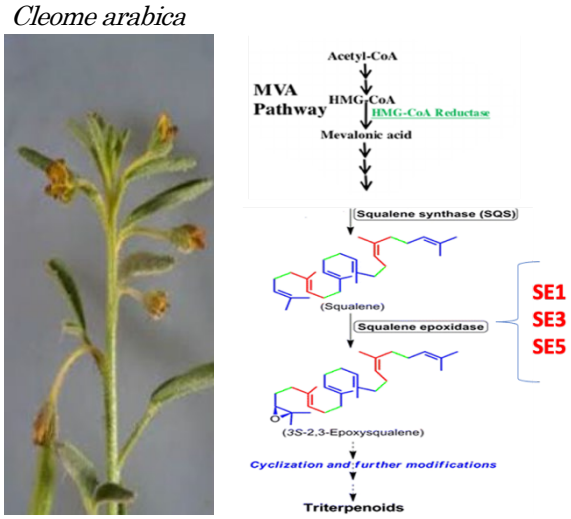

Fig. 1. Predicted biosynthetic pathway of terpenoids in *Cleome arabica*

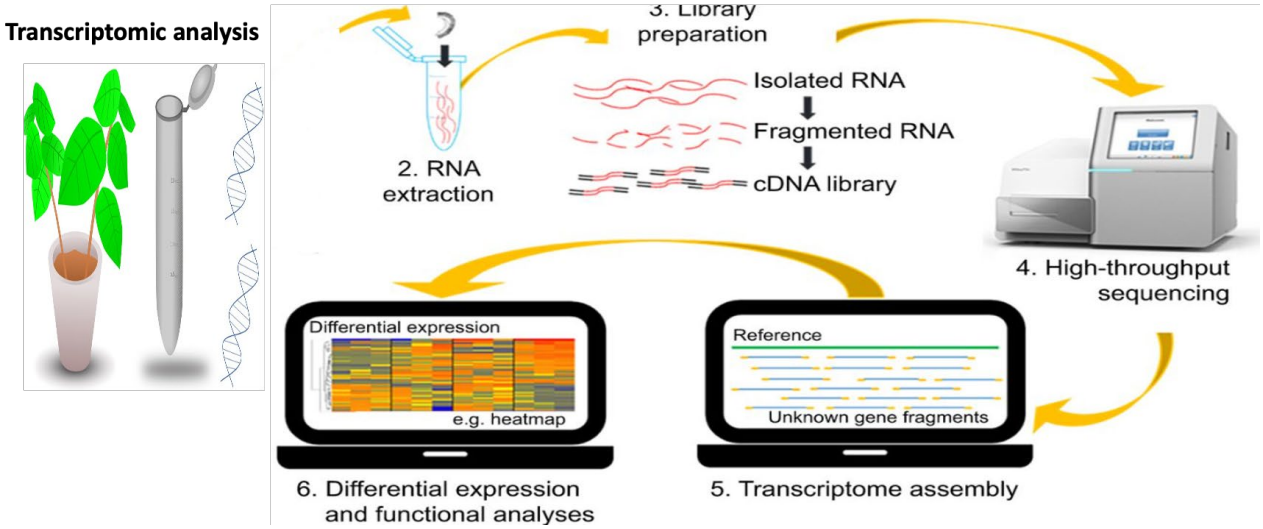

Fig.2. Transcriptomic analysis of plant tissues of *C. arabica*

The transcriptomic data lead us to identify the candidate genes involved in triterpene biosynthesis. The predicted biosynthetic pathway of terpenoids includes the squalene synthase (SQS), squalene epoxidase (SE), oxidosqualene cyclases (OSC), and dammarenediol-II synthase (DDS), and putative orthologs were identified from the unigene set of *C. arabica* (Fig. 1). The gene expression of them showed significant difference among different tissues of Cleome. The CaSQS

was highly expressed in roots compared to the leaves, stems, flowers and siliques, while CaSE expression showed significant difference between CaSE1, CaSE3, and CaSE5. Tissue-specific expression of CaSQS and CaSE were analyzed based on the transcriptomic data and RT-PCR in different tissues of *C. arabica.* Recent analyses of SQS enzymes from several plant species have shown that SQS proteins have two transmembrane regions in the carboxy-terminal, suggesting they are membrane proteins residing on endoplasmic reticulum membrane. In *N. benthamiana* and *C. arabica*, GFP-fused CaSQS and CaSE were localized in endoplasmic reticulum membrane. To demonstrate protein function, CaSQS was tried to be expressed in E. coli. The full-length clone was not properly expressed but a truncated form (33-329 aa) was successfully expressed in E. coli. The enzyme activity of the recombinant CaSQS was confirmed by the measurement of its product by GC-MS analysis.

On the other hand, the transient expression assay was performed in *Nicotiana benthamiana* leaves with a combination of upstream and downstream pathway genes in order to characterize terpenoids genes. The CaHMGR expression in *N. benthamiana* effectively increased squalene and 2,3-oxidosqualene, while the expression of CaHMGS and CaSQS did not make any change. In this project, the chemical profile of dammarane type triterpene was determined also in different tissues of *C. arabica* through GC-MS analysis.

Establishment of method to manipulate metabolic pathway by dsRNA application to plant

To downregulate the expression of genes of interest, microRNAs (miRNAs) were employed in this study. As a proof-of-concept, we applied an amiRNA-expressing vectors for CaSQS and CaSE as target genes to downregulate their function in *C. arabica* leaves by Agro-infiltration (Fig. 3). The RT-PCR analysis showed that the gene expression of CaSQS and CaSE in *C. arabica* were significantly downregulated after 5 days. Even though further analysis of metabolite accumulation is needed, this result opens up a new avenue to manipulate metabolite pathway exogenously in plant.

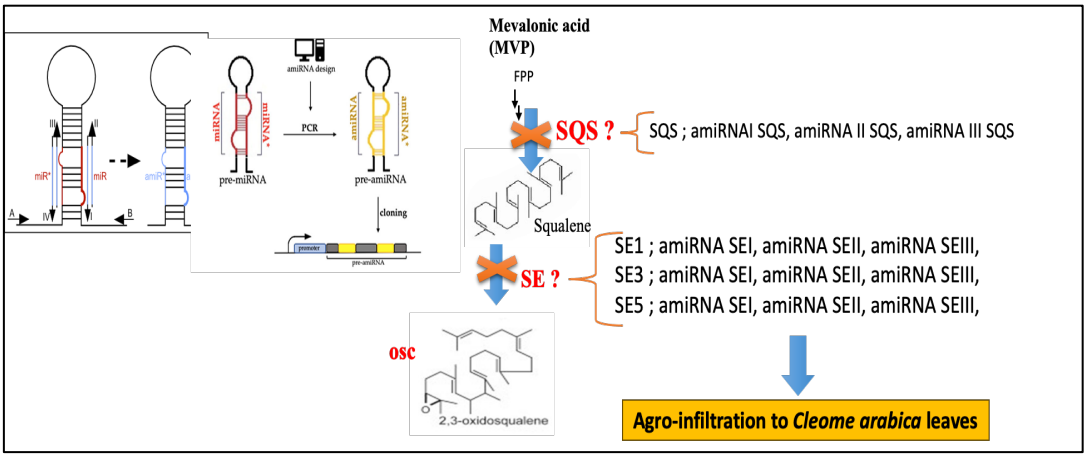

Fig.3. Designed artificial microRNA for target gene through the WMD3 web microRNA designer

Conclusion

The medicinal plant *Cleome arabica* L produces high-value terpenoids such as pentacyclic triterpenes and dammarane triterpenes. Squalene synthase (SQS) and squalene epoxidase (SE) catalyze key steps in the biosynthesis of cyclic terpenoids, but neither enzyme has yet been characterized in *C. arabica*. Genomic analysis has shown the presence of one (CaSQS) and three (CaSE1–3) orthologous genes. The CaSQS and CaSE1-3 proteins are localized in the endoplasmic reticulum membrane and the enzymatic functions of CaSQS was confirmed by *in vitro* activity assays. The functions of the upstream and downstream pathway genes of terpenoids biosynthesis were further characterized through the transient over-expression in *N. benthamiana* leaves. AmiRNA application successfully downregulated CaSQS and CaSE, indicating that this technology could optimize the metabolic flux toward specific terpenoids during development of *C. arabica*.

（2）主な研究発表（雑誌論文、学会、集会、知的財産権等）/ Main Research Publications

We are planning to publish at least two papers devoted to the identification of terpenoids pathway in *Cleome arabica.*

（3）その他/ Remarks

No Remarks