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研究課題名（和文）ポプラにおける菌根形成に関与するmiRNAの分子メカニズムの解明

研究課題名（英文）Molecular mechanism of poplar miRNAs involved in ectomycorrhiza formation

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研究成果の概要（和文）：miRNA-seqにより、Cenococcum geophilumに感染したポプラの根で発現が変動している51個のmiRNAとそれに対応する2043個のターゲット配列を同定した。次に、psRNATargetにより、51個のmiRNAの標的配列を予測した。miRNAs-seqとpsRNATargetによる予測はさらにデグラドームシーケンシングとRT-qPCRによって確認された。GOおよびKEGGエンリッチメント解析は発現が変動したmiRNAの標的遺伝子がフェニルプロパノイド生合成経路、イソキノリンアルカロイド生合成経路そしてアポプラスト経路などを通してECM共生系を制御していることを示している。

研究成果の学術的意義や社会的意義
The research demonstrates that Populus miRNAs and their target sequences are essential to ectomycorrhizal symbiosis, which plays key roles in forest ecosystems and carbon cycle. The results may supply some new insights about how to manage and protect forests.

研究成果の概要（英文）：We used the miRNA sequencing to identify 51 differentially expressed miRNAs and corresponding 2043 targets in the roots of Populus tomentosa colonized by Cenococcum geophilum. Then we predicted the target sequences of 51 miRNAs with psRNATarget. The prediction of miRNAs-seq and psRNATarget were further confirmed by degradome sequencing and RT-qPCR analysis. GO and KEGG enrichment analysis indicated that the target genes of some differentially expressed miRNAs regulate the ECM symbiosis through the pathways of phenylpropanoid biosynthesis, isoquinoline alkalold biosynthesis, and apoplast, etc. This work indicates that Populus miRNAs and their target genes are an important part of the regulatory network leading to ECM symbiosis development.

研究分野：Forest Science

キーワード：MiRNA sequencing　Degradome sequencing　Ectomycorrhiza　Populus tomentosa　RT-qPCR　Phenylpropanoid　Cenococcum geophilum

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様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９（共通）

１．研究開始当初の背景

菌根菌は、地球上の 80%の陸上植物と菌根を形成している（Brundrett and Tedersoo, 2018, New Phytol）。菌根菌は菌根の形態から、内生菌（AM 菌）、外生菌（ECM 菌）、エリコイド型菌、ラン型菌根、に分けられるが、ほとんどは AM 菌と ECM 菌である。菌根菌は宿主の水と養分の吸収を助け、宿主に生物・非生物学的なストレスへの耐性を付与する。その代わりに、宿主から光合成産物を得る（Garcia et al., 2017, Plant Physiol）。この共生系は、農業だけでなく自然生態系においても重要である。このため、菌根菌の共生の進化、菌根形成の分子メカニズムついて、研究が行われてきた。

菌根形成のメカニズムについては、遺伝学的研究により、AM 菌中のストラゴラクトンの合成に関わる 2 つの酵素（CCD7、CCD8）が、菌根形成のシグナルを誘導し、根の分枝の促進することが明らかにされている（Vogel et al., 2010, Plant J; Schmitz et al.,2014, J Integr Plant Biol）。また多くの菌根菌において、chitin synthase Nod C、Nod B、acyltransferase Nod A 遺伝子は、菌根形成のシグナル分子 lipochito-oligosaccharides（Myc-LCOs）の合成をする重要な酵素の遺伝子であることがわかっている（Martin et al., 2008, 2010, Nature）。一方、ECM 菌については、Jasmonic acid repressor JAZ6 と coronatine-insensitive 1 の相互作用によって、ジャスモン酸の発現を抑制し、菌根形成を促進したとの報告がある（Plett et al., 2011, Curr Biol; 2011, PNAS）。しかし、EMC の菌根形成のメカニズムについてはまだ十分に理解が進んでいない。

miRNA は 20-24 塩基の長さからなる非コード RNA で、およそ 25 年前に発見された。miRNA は、II 型 RNA ポリメラーゼによって転写され、転写後、2 種類の RNase III（Drosha, Dicer）によって切断されることで、pre-miRNA を経て、2 本鎖の成熟 miRNA となる（Bushati et al., 2007, *Annu Rev Cell Dev Biol*）。成熟 miRNA は、RNA 誘導サイレンシング複合体（RNA-induced silencing complex）となり、miRNA と相補的な配列（標的部位）を持ったターゲット遺伝子の翻訳を抑制する。miRNA と標的部位の配列が相補的に完全に一致または相同性が高いとき、ターゲットの遺伝子の転写産物である mRNA は分解される（Zhou et al., 2018, *Signal Transduct Target Ther*）。また、相補的な結合が不完全な場合であっても、mRNA の転写は抑制される。この miRNA による遺伝子の制御は、例えば細胞の増殖、分化、オートファージ、代謝と免疫など、多くのプロセスに関与している。miRNA は、植物と菌根菌との相互作用にも関与することが示されており、特に AM 菌において詳細に調べられてきた（Bonfante and Genre, 2010, *Nat Commun*）。例えば、トマトではゲノム配列から miRNA とそのターゲットの遺伝子の予測が行われ、またマイクロアレイを用いて 5 つの miRNA が AM 菌との菌根形成に関連することが示された（Gu et al., 2010, *Physiol Plant*, Gu et al., 2014, *J Integr Plant Biol*）。また次世代シーケンサーを用いることで（miRNA-seq）、網羅的に miRNA を検出できるようになった（Devers et al., 2011, *Plant Physiol*）。近年では、ウマゴヤシの miR171h が、根粒の遺伝子（*Nodulation Signaling Pathway2*）の発現を制御することで、AM 菌との共生に関与することまで明らかになっている（Lauressergues et al., 2012, *Plant J*）。

一方、***ECM 菌では miRNA が菌根形成にどう作用するのか、ほとんどわかっていない***。わずか、ゲノム配列からの miRNA の探索とそのターゲット遺伝子の予測および miRNA-seq を行った研究（Mewalal et al., 2019, *Front Microbiol*）にとどまる。そこで本研究では、***どのような miRNA が ECM 菌において菌根形成と関連するのか***、またその ***miRNA がどの遺伝子の発現を制御しているのか***、さらに、***その遺伝子の働きを明らかにする***ことで、***ECM 菌における菌根形成の分子メカニズムの解明を試みる***。

２．研究の目的

In this study, we will use *Populus tomentosa* as the representative host plants and *Cenococcum geophilum* as the representative ECM fungi to research the molecular mechanism of ectomycorrhiza (ECM) formation. (1) We will clarify which *Populus* miRNAs are associated ECM formation. (2) We will attempt to make sure which genes are the targets of *Populus* miRNAs involved in the ECM formation. (3) We will find out the expression patterns of *Populus* miRNAs and their targets in ECM formation. (4) Hence, we will select several miRNAs and their target genes to research their functions in ECM formation. (5) Finally, we hope to establish the regulatory network of miRNA-mediated mRNA cleavage targets in ECM formation.

３．研究の方法

(1) Inoculation with *Cenococcum geophilum*

After one month of culture, six independent wild-type *P. tomentosa* seedlings (~12 cm) were used for inoculation experiments. The isolates of *C. geophilum* pre-cultured in agar modified MMN medium with a sterile cellophane membrane for 30 days. The agar block (~ 4 cm^2) containing *C. geophilum* mycelium was inoculated around the roots of *P. tomentosa* seedlings in the experimental group, while the empty agar block was inoculated in the control group.

(2) Construction of miRNA libraries and deep sequencing

5 ug high quality RNA for each sample was sent to the Biomarker Technologies Corporation for library construction and deep sequencing.The raw data was obtained after sequencing on the Illumina NovaSeq 6000 platform, and then adapter and low-quality data in raw data were removed to obtain the clean data. Clean reads were mapped to remove rRNA, tRNA, snRNA, snoRNA, and repeated sequences. And then the rest reads which containing miRNAs were mapped to the reference genome *Populus_trichocarpa*.v3.0 with Bowtie (Langmead *et al*., 2009, *Genome Biol*), and reads with positions were called mapped reads.

Known miRNAs were identified by comparing mapped reads with mature miRNA in miRBase (v22) database. And the remaining reads were analyzed by miRDeep2 (Friedlander *et al*., 2012, *Nucleic Acids Res*) to predict novel miRNAs based on specific species. Differentially expressed miRNAs were detected by DESeq2 (Love *et al.*, 2014, *Genome Biol*).

(3) Predictions and functional annotation of target genes of MiRNAs

Putative targets of miRNAs were predicted using miRNA sequencing. The target genes of miRNAs were predicted and aligned based on sequences of known miRNAs, novel miRNAs, and genomic sequences of *P._trichocarpa* by TargetFinder and psRNATarget.

Sequences of all targets were BLAST against NR, Swiss-Prot, GO, COG, KEGG, KOG, and Pfam database to obtain their annotations basing on homology alignment. Hence, the target genes were annotated to GO terms and KEGG pathways.

(4) Construction of degradome libraries and deep sequencing

An aliquot of the RNA sample for miRNA sequencing was used for generating degradome libraries. Poly(A) RNA was isolated from total RNA and purfied using the Oligotex mRNA Kit (Qiagen, Hilden, German). 1 ug of poly(A) RNA for each sample were sent to LC-Bio Technologies (Hangzhou, China) for the degradome library construction, quality accessment, and deep sequencing.

After sequencing the degradome library, target transcripts of miRNAs were predicted using the CleaveLand program. Needle program in the EMBOSS package was used to extract all sequences which

matching to the sequences of mature miRNAs. Arrays was scored according to the plant miRNA-target interaction criteria (Allen *et al*., 2005, *Cell*). The fraction can't exceed the set threshold, and the 10th nucleotide at the 5' end of the degradome sequence paired with miRNA was preserved.

(5) RT-qPCR analysis

An aliquot of the RNA sample for miRNA sequencing and degradome sequencing was used for RT-qPCR analysis. The first Strand cDNA Synthesis SuperMix was used to synthesize the first-strand cDNA for reverse-transcription PCR. The RT-qPCR assay was performed in accordance with a previously published protocol (Li *et al*., 2018, *Front Plant Sci*). The transcript levels of target genes of *miR164/319/396/397/398* were determined using gene-specific primer pairs. The relative expression levels of mature miRNAs were assayed via the highly sensitive stem-loop RT-qPCR method. The U6 snRNA was used for data normalization of *miR164/319/396/397/398* expression.

４．研究成果

(1) A total of 432 *Populus* miRNAs were identified after miRNA sequencing, of which 276 were known miRNAs and 156 were novel miRNAs. Meanwhile, 51 *Populus* miRNAs were significantly induced after inoculation with *C. geophilum*, including 13 novel miRNAs. Expression pattern analysis showed that 25 miRNAs were significantly up-regulated, while 26 miRNAs were down-regulated in ECM roots.

(2) Compared with other differentially expressed miRNAs (DE-miRNAs), the changes of *Populus miR164/319/396/397/398* and novel *miR44* were more obvious. Therefore, we analyzed their expression with the stem-loop RT-qPCR method. The expression of *miR164/319/397/398* was significantly up-regulated after inoculation of *C. geophilum* on the roots of *P. tomentosa*, whereas the expression of 396/novel miR44 was down-regulated. The results were consistent with miRNA-seq analysis.

(3) Bioinformatics prediction and degradome analysis indicated that 6,672 genes were the target genes of 432 *Populus* miRNAs, of which 2,043 genes were the predicted target genes of DE-miRNAs. To further confirm the results of degradome-seq, RT-qPCR assay was introduced to check the expression of predicted target genes of DE-miRNAs. The expression of targets of *miR164/319/397/398* was significantly down-regulated, whereas the expression of target genes of miR396/novel miR44 was significantly up-regulated. The expression patterns of target genes had the opposite trends with their corresponding DE-miRNAs.

(4) KEGG enrichment analysis showed some targets were mainly enriched in the processes of isoquinoline alkaloid biosynthesis, phenylpropanoid biosynthesis, SNARE interactions in vesicular transport, and arginine and proline metabolism, etc. For phenylpropanoid biosynthesis, the lignin enzyme genes *PAL* and *4CL*, cytochrome P450 family members, UDP-glucose 6-dehydrogenases (UGD), and peroxidases (PODs) were significantly enriched, which are responded to the known *miR393/394/398/1444/6457* and novel *miR44/48/80*. For isoquinoline alkaloid biosynthesis, many polyphenol oxidases (PPOs) and novel *miR1444* were the main factors that regulating ECM formation.

(5) We also used GO enrichment analysis to analyze DE-miRNAs and their target genes. For cell component aspect, the targets mainly participated in the processed of aopolast, plasmodesma, chloroplast thylakoid membrane, and NLS complex, etc. Meanwhile, more than 30 laccases (LACs) were enriched in the aopolast, and they were the targets of *miR396/397/397/408* and novel *miR44/48/80*. A series of RLK proteins were enriched in the process of plasmodesma, of which were the targets of *miR162/398/482* and novel *miR44/58/80*. All LACs were up-regulated after inoculation of *C. geophilum*, while all RLK proteins in plasmodesma were down-regulated. For molecular function, LACs and their corresponding miRNAs

(*miR396/397/397/408* and novel *miR80*) were enriched in the coppering ion binding, ferroxidase activity, and hydroquinone oxygen oxidoreductase activity. PPOs and novel *miR1444* were enriched in the processes of catechol oxidase activity. For biological process, the LACs were enriched in iron ion transport, lignin catabolic process, and iron ion homeostasis, while the PPOs were enriched in the pigment biosynthetic process.

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2．論文標題	5．発行年
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3．雑誌名	6．最初と最後の頁
Plant Cell and Environment	3015-3033
掲載論文のDOI（デジタルオブジェクト識別子）	査読の有無
10.1111/pce.14127	有
オープンアクセス	国際共著
オープンアクセスではない、又はオープンアクセスが困難	該当する

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10.1007/s00572-022-01078-1	有
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〔図書〕　計0件

〔産業財産権〕

〔その他〕

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6．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

7．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

8．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関