論文の内容の要旨

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論文題目

^{13}C labeling-based approach towards unlocking of diterpenoid phytoalexins biosynthetic pathways in rice

（^{13}C 標識ジテルペン炭化水素の投与によるイネのファイトアレキシン生合成経路の解明）

Introduction

In rice (*Oryza sativa*), 17 compounds have been identified as phytoalexins from leaves that were either infected with the *Magnaphorthe oryzae* or subjected to UV irradiation. Other elicitations, such as heavy metal treatment, also can induce phytoalexins production in rice. All these phytoalexins, except for the flavanone sakuranetin, are diterpenoid in nature, and can be classified into five groups based on their carbon frameworks: phytocassanes A-F, oryzalexins A-F, momilactone A and B, oryzalexin S, and casbane-type oxodepressin. Great efforts to disclose the whole biosynthetic pathway of the phytoalexins, momilactones and phytocassanes, have been made up to now. It is known that the majority of their biosynthetic genes are clustered in chromosomes 2 and 4. Genes encoding cytochrome P450 monooxygenases in the each cluster are shown to be involved in the oxidation step of *syn*-pimara-7,15-diene to *syn*-pimara-7,15-dienoic acid (*CYP99A2* and *A3*) in momilactones biosynthesis and the oxidation step of *ent*-cassa-12,15-diene to phytocassanes (*CYP71Z7*, *CYP76M7*, *M8*). Several lines of evidence from both *in vivo* and *in vitro* experiments have shown that many P450 genes are putatively responsible for the biosynthesis of diterpenoid phytoalexins in rice. However, there are still substrate-enzyme specificities among P450s that remain unclear and need to be explained in order to understand phytoalexins biosynthesis. A feeding approach with stable isotope (e.g., ^{13}C) will be

an effective method to trace as-yet-unknown precursors or enzyme specific substrates derived from known compounds such as *ent*-cassa-12,15-diene and *syn*-pimara-7,15-diene, the diterpene hydrocarbons for phytocassane (A-F) and momilactone (A and B), respectively. In this study, we synthesized ^{13}C-labeled diterpene compounds *in vitro* enzymatically, and performed feeding experiments for elucidating the possible diterpene biosynthetic pathways occurring *in planta*.

Chapter 2. Enzymatic production of ^{13}C-labeled diterpene hydrocarbons

Toward unlocking the biosynthetic pathway of rice diterpenoid phytoalexins, we first generated ^{13}C-labeled diterpene hydrocarbons used for feeding experiments in following chapters. The fully ^{13}C-labeled *ent*-cassa-12,15-diene and *syn*-pimara-7,15-diene, either of which is the precursor of phytocassanes or momilactones, were enzymatically synthesized from [U-$^{13}C_{20}$] geranylgeranyl diphosphate (GGDP). The synthesis of *ent*-cassa-12,15-diene from GGDP required two diterpene cyclases, OsCPS2 and OsKSL7 involving in *ent*-CDP synthesis from GGDP and *ent*-cassa-12,15-diene synthesis from *ent*-CDP, respectively. The synthesis of *syn*-pimara-7,15-diene from GGDP can be performed by using diterpene cyclase, HpDTC1. HpDTC1 has been identified from the moss, *H. plumaeforme*, and is involved in sequential two-step cyclizations from GGDP to *syn*-pimara-7,15-diene via *syn*-CDP. The yields of both labeled *ent*-cassa-12,15-diene and *syn*-pimara-7,15-diene from ^{13}C-mevalonate reached approximately 90% and 66%, respectively, indicating that both enzyme reactions are highly efficient for the synthesis of labeled substrates. GC-MS and ^{13}C-NMR spectra validated the production of the [U-$^{13}C_{20}$] *ent*-cassa-12,15-diene and [U-$^{13}C_{20}$] *syn*-pimara-7,15-diene from [U-$^{13}C_6$] mevalonate as fully labeled compounds. Thus, we were able to generate biosynthetic precursors of diterpenoid phytoalexins labeled with ^{13}C isotope, capable of chasing metabolites leading to diterpenoid phytoalexins.

Chapter 3. Establishing feeding platforms of the labeled substrates incorporated into diterpenoid phytoalexins

To investigate the bioconversion of the diterpene hydrocarbon precursor into phytoalexins *in planta*, both the labeled precursors were fed into leaf disks (6 mm diameter) or young leaves (30 mm in length) of wild-type rice plants (*O. sativa* 'Nipponbare'), and analyzed by LC-MS/MS, to

confirm the presence of ^{13}C-labeled compounds. After the application of [U-$^{13}C_{20}$] *syn*-pimara-7,15-diene, [U-$^{13}C_{20}$] momilactone A and [U-$^{13}C_{20}$] momilactone B were detected to be accumulated in the leaf disk. Successful [U-$^{13}C_{20}$] *ent*-cassa-12,15-diene feeding experiment on rice leaves also gave the direct evidence of biotransformation of ^{13}C-phytocassanes from [U-$^{13}C_{20}$] *ent*-cassa-12,15-diene. These results suggest the conversion of momilactones and phytocassanes from *syn*-pimara-7,15-diene and *ent*-cassa-12,15-diene, respectively, in rice plants. This implied that the biosynthetic pathways of diterpenoid phytoalexins from these diterpenes are definitely active in rice.

Chapter 4. Investigation of diterpene intermediates in the biosynthetic mutants of cytochrome P450 monooxygenases for diterpenoid phytoalexins

Characterization of *cyp71z7* T-DNA insertion mutant and *CYP76M7M8-RNAi* plants have previously shown that cytochrome P450 monooxygenases genes *CYP71Z6/Z7* and *CYP76M7/M8* in the phytocassane biosynthetic gene clusters are involved in oxidation steps of diterpenoid phytoalexins biosynthesis. Therefore, possible intermediates accumulated in these CYP-modified plants were further investigated by using our feeding system. The application of [U-$^{13}C_{20}$] *ent*-cassa-12,15-diene to *cyp71z7* mutant revealed that the accumulation of C2-oxygenated types of phytocassanes (^{13}C-phytocassane A, B and D) was obviously repressed whereas the accumulation of ^{13}C-labeled C2-non-oxygenated types of phytocassanes (^{13}C-phytocassane C, E) was enhanced compared with wild-type plants. Furthermore, GC-MS analysis confirmed two intermediates in *cyp71z7* mutant, 1-deoxyphytocassane C and 2-deoxyphytocassane A, both of which were proposed as the precursor of phytocassanes.

On the other hand, none of the ^{13}C labeled phytocassanes were found in *CYP76M7/M8-RNAi* lines fed with [U-$^{13}C_{20}$] *ent*-cassa-12,15-diene, implying that metabolic flow from *ent*-cassa-12,15-diene might stuck and intermediates would be accumulated in this RNAi line. GC-MS analysis showed that two distinctive peaks dominant in *CYP76M7/M8-RNAi* were detected and low level of ^{13}C isotopic signals were monitored on the mass fragmentation. Consequently, two intermediates, 3α-hydroxy-*ent*-cassadiene and 3α-hydroxy-*ent*-cassadien-2-one, were discovered by comparing with reference compounds *in vitro* assay.

Previous study has shown that CYP76M8 has enzymatic activity for hydroxylation at

C6-position of pimaradiene, a momilactone precursor. To investigate this point, [U-$^{13}C_{20}$] momilactone A accumulation level in *CYP76M7/M8-RNAi* lines and wild-type rice plants were examined by LC-MS/MS after feeding of [U-$^{13}C_{20}$] *syn*-pimara-7,15-diene. Accumulation level of both non-labeled ^{13}C-labeled momilactones apparently decreased in the *CYP76M7/M8-RNAi* line compared to that in wild-type plants, suggesting that CYP76M8 is most likely to be involved in both phytocassanes and momilactones biosynthetic pathways. Further GC-MS analysis revealed that fully ^{13}C-labeled compounds derived from [U-$^{13}C_{20}$] *syn*-pimara-7,15-diene with unknown mass spectrum decreased in the *CYP76M7/M8-RNAi* lines. Although the structural information of this unknown compound is still elusive, these results demonstrate that enzymatically synthesized [U-$^{13}C_{20}$] diterpene substrates are a powerful tool for chasing endogenous metabolites without suffering from unavoidable dilution with natural abundance.

Chapter 5. The application of ^{13}C-labeled diterpene hydrocarbons to other plants

It has recently been reported that some of the plants, other than rice can produce two distinct types of phytocassanes (phytocassane C and E) or momilactones. Further application of this feeding method, using [U-$^{13}C_{20}$] *syn*-pimara-7,15-diene and [U-$^{13}C_{20}$] *ent*-cassa-12,15-diene in newly found phytoalexins-producing non-model plants, the moss *Hypnum plumaeforme* and the nearest out-group of *Oryza* species *Leersia perrieri*, respectively, resulted in detection of bioconversion of these labeled substrates into phytoalexins in these plants. These results demonstrate that enzymatically synthesized [U-$^{13}C_{20}$] diterpene substrates are a powerful tool for chasing endogenous metabolites without dilution with naturally abundant unlabeled compounds.

Reference

Zhongfeng Ye, Kazuya Nakagawa, Masahiro Natsume, Hideaki Nojiri, Hiroshi Kawaide*, Kazunori Okada* Biochemical synthesis of uniformly ^{13}C-labeled diterpene hydrocarbons and their bioconversion to diterpenoid phytoalexins in planta. *Bioscience Biotechnology and Biochemistry.* 2017, Feb. 6:1-9