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研究課題名（和文）Elucidation of molecular mechanisms for transduction of jasmonate signal to fertility in rice

研究課題名（英文）Elucidation of molecular mechanisms for transduction of jasmonate signal to fertility in rice

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研究成果の概要（和文）：イネの花器官の植物ホルモンの解析より、開花期にJA-Ile量が最大となることを見出した。マイクロアレイ解析により同定した、花におけるJAシグナルの下流遺伝子のいくつかはシグナル制御因子であった。そのなかには、イネの稔性のマスター制御因子候補となる機能未知のNAC、MADS-boxやAP2遺伝子が含まれていた。さらに我々は、イネの栄養組織においてJAシグナルで制御される防御関連二次代謝物が、生殖成長後期においてはJAシグナルとは独立して制御さることを明らかにしつつある。得られた成果は、植物が自然界において最適に適応し成長する上で重要な、成長と防御の間のシグナルクロストークに新しい洞察を与える。

研究成果の学術的意義や社会的意義
植物の稔性は生産性を大きく左右する。我々は防御応答への関与が知られている植物ホルモンであるジャスモン酸が、どのように花の発生を制御するのか解析を進めた。ジャスモン酸が制御する生殖とファイトアレキシン産生の間の分子クロストークの潜在的な側面をひも解いている。主な社会的意義としては、地球温暖化の進行や気候変動によって引き起こされる劣悪環境下においても持続的に収穫が可能な、高収量でストレス耐性を持つ次世代作物の作出において、得られた知見が利用されることが期待できる。

研究成果の概要（英文）：We characterized in detail hormonal contents in rice flowers, finding that high levels of JA-Ile coincide with anthesis stage in the rice monocot model. Next, we identified multiple novel targets of JA signaling in flowers by microarray analysis, some of which belong to regulatory class genes. Out of them, novel JA-controlled NAC, MADS-box and AP2 genes of yet unknown functions were depicted as potential master regulators of fertility in rice. Furthermore, we make progress in understanding of metabolic defense in rice that is controlled by JA pathway in vegetative tissues but apparently becomes uncoupled from JA signal during later reproductive stages. Overall, our research provides advances in understanding of cross-talk between growth and defense that is important for achieving optimal fitness and growth of plants in nature.

研究分野：応用分子細胞生物学

キーワード：rice　flower development　fertility　defense crosstalk　jasmonic acid　イネ　植物ホルモン

様　式　C－１９、F－１９－１、Z－１９、CK－１９（共通）

１．研究開始当初の背景（Introduction)

Oxylipins jasmonic acid (JA) and its derivative jasmonoyl-L-isoleucine (JA-Ile) are essential hormonal signals for activation of innate defense responses in plants. Both hormones rapidly accumulate in higher plants after wounding and herbivore feeding, and during necrotrophic pathogen attack. Jasmonate accumulation in the leaves leads to activation of genes and biosynthesis of phytoalexins, and other defense compounds. Major principles of jasmonate defense signaling in plants have then been elucidated at molecular level in 2007 showing that active jasmonate, jasmonoyl-L-isoleucine (JA-Ile) triggers degradation of repressors of multiple defense response genes in plants. Strikingly, most plants defective in jasmonate biosynthesis, or insensitive to this hormone, are also impaired in reproduction (sterile). It has been confirmed in various mutants in Arabidopsis (*coi1*; *fad3fad7fad8*; *dad1*; *opr3*; *dde1*), tobacco (*coi1*), and in two monocot plant species, rice (*hebiba* or *cpm2*; *jar1*) and maize (*ts1*; *opr7opr8*). Flower-development phenotypes associated with JA deficiency in both dicots and monocots demonstrate universal evolution-conserved role of jasmonates in plant reproduction, however molecular targets of jasmonate action in flowers remain poorly understood. In this research, we focused on understanding of jasmonate function in reproduction and its crosstalk with defense using rice model system.

２．研究の目的（Purpose of research)

We aimed to understand functional role of oxylipins in rice reproduction. In practice, we used wild type and jasmonate biosynthesis-deficient rice plants and identified differentially expressed genes in flowers before and during anthesis. The function of jasmonate-dependent candidate genes in flowers was then subjected to analysis at molecular level using transgenic plants silenced in the expression of these genes. In parallel, we studied multitude of defense functions of jasmonates to understand the basis of molecular crosstalk of defense and development controlled by jasmonate pathway.

３．研究の方法（Methods)

Two jasmonate deficient rice lines were used in this research (*0sjar1*, *hebiba*). Two independent TOS17 mutants of *0sjar1* from NIAS resources (NG8484, NC0364) were selected based on our previous research. Plant propagation and sample collection were performed in the field and/or greenhouse. Main sites and timing of jasmonate biosynthesis in rice flowers were determined by LC-MS/MS. Gene expression patterns of known regulators of flowering (MADS box) was determined by quantitative RT-PCR. Global gene expression patterns were examined by microarray system (Agilent oligoprobe microchip) in flower tissues isolated at early stages of flower development that are characteristic by their high levels of jasmonic acid/JA-Ile. Expression of differentially expressed flower genes putatively associated with JA function in flowers was validated by quantitative RT-PCR. Gene fragments of three candidate genes were cloned and transgenic plants silenced in these gene products were constructed by Agrobacterium transformation method. T1 generation of transgenic plants is continuously scored for potential phenotypes associated with fertility in correlation with gene silencing levels in each independent transgenic line (>30 each). In parallel, levels of typical jasmonate-regulated defense metabolites (phenolamides and diterpene phytoalexins) were examined in flowers and other tissues by LC-MS/MS. GC-MS was further used to characterize volatile emissions from mutant plants as these compounds are also known to be dependent on jasmonate signaling.

４．研究成果（Results)

At first, we examined jasmonate levels in developing rice panicles. As jasmonates accumulated mainly in early stages of panicle development (until anthesis), we used these stages for differential gene expression analysis. Microarray experiment resulted in the identification of multiple genes deregulated in the absence of JA signal in the flower tissues. As these genes are primary candidates for control of fertility in rice, their function became the main subject of this study as well as it provided strong leads for our future investigations.

(1) Jasmonate deficiency causes sterility in rice

Before this project, we only used *0sjar1* mutant plants which are sterile due to lack of jasmonic acid modification to JA-Ile conjugate, substance known as main active form in jasmonate signaling. Previously, we reported that *0sjar1* mutant plants lack most of its JA-Ile in the leaves; however, as other enzymes can also conjugate JA to JA-Ile in rice, we established another model for parallel investigations using *hebiba* mutant that lacks

functional allele of ALLENE OXIDE CYCLASE (AOC) and these plants, therefore, cannot accumulate any JA or JA-Ile. With permission of Professors M. Riemann and P. Nick, *hebiba* seeds were obtained from Prof. K. Okada group at Tokyo University in Japan. We confirmed that, as reported previously, *hebiba* plants grown in the laboratory are sterile and unable to set seeds, showing similar phenotype as *Osjar1* mutant. Both *Osjar1* and *hebiba* were then used in parallel to investigate the jasmonate role in fertility and defense of rice plants as proposed. Remarkably, *hebiba* plants, in contrast to *Osjar1,* were unable to grow in the paddy field due to high rate of pathogen infections, severe necrosis and wilting.

(2) Accumulation patterns of plant hormones in developing rice panicles

Using both *Osjar1* and *hebiba*, we determined the accumulation of JA, JA-Ile, their common precursor metabolite OPDA, and their degradation products OH-JA, OH-JA-Ile, COOH-JA-Ile. In addition, we also investigated abscisic acid and salicylic acid levels in developing panicles. As expected, both mutants showed highly reduced JA-Ile contents. JA levels were diminished in *hebiba* but increased in *Osjar1* due to block in conversion of JA to JA-Ile. Most importantly, we found that both wild-types corresponding to *Osjar1* and *hebiba* mutants, Nipponbare and Nihonmasari, respectively, accumulated high levels of JA and JA-Ile in panicles at early stages of development that was before and during anthesis (maximum levels 50-70 ng/gFW). After anthesis, oxylipin hormone levels rapidly declined. This supported the original notion of essential role of JA to be prior and/or during anthesis, which then directly links to fertility and seed development of rice. For the first time, we also show that hydroxylated jasmonate degradation products co-appear with the active forms in flowers, suggesting that JA-Ile levels are actively controlled by co-activated hydroxylation activity of cytochrome P450 enzymes in rice reproductive tissues.

(3) Analysis of MADS-box gene family in *Osjar1* and *hebiba* rice mutants

Prior to global scale gene expression experiments, we first examined regulation by jasmonic acid of several critical transcriptional regulators in flower development known as MADS-box genes using quantitative RT-PCR. Surprisingly, none of the examined genes (MADS1, 2, 3, 4, 13, 58, DL) expression was strongly impaired in its expression in jasmonate-deficient rice flowers. Using public database RiceXPro with tissue specific expression data, we also selected several other genes specifically expressed in rice flower parts (for example, Os04g0435100, Os04g0629300, Os05g0509500). Especially in *Osjar1* mutant, the expression of these genes was upregulated after anthesis, such as the flowers were arrested at anthesis and could not proceed to the next stage of seed development. It suggested that JA-Ile is a critical developmental switch that ensures post-anthesis transition and subsequent development in rice but this process does not link to known morphoregulators in plants, such as MADS-box genes.

(4) Global gene expression in jasmonate-deficient panicles

Anthesis stage was selected for microarray experiment due to presence of highest level of jasmonates found in these tissues that is likely to maximally affect gene expression. Two independent TOS17 mutants of *Osjar1* from NIAS resources (NG8484, NC0364) were used to assure high specificity of obtained data. To compensate for expected biological variation in experiment, 4 biological replicates of anthesis stage NC0364 and NG8484 panicles collected in the field were hybridized with respective controls to obtain dataset of differentially expressed genes controlled by JA-Ile. As a result, we found 487 genes that were more than 2-fold commonly downregulated and 242 that were more than 2-fold commonly upregulated in the absence of JA-Ile, suggesting that moderate to large number of genes is transcriptionally controlled by JA-Ile during flower development in rice. Out of these genes, many belong to transcriptional regulators and thus classify as potential targets of jasmonate regulation. Other genes belong to enzymes and proteins that putatively play important structural and metabolic functions during flower development. With such extensive number of potentially important genes, we first focused on highest regulated transcription factors that may be functionally linked to JA-Ile hormonal function.

(5) Validation of microarray results and construction of transgenic plants

In order to validate microarray results, cDNA from four flowering stages (*Osjar1*) and five stages (*hebiba*) was used. In principle, good correlation between microarray and RT-PCR data was found and further research was carried out with three validated potential regulators, one NAC, one MADS-box type and one AP2 type gene. All genes were transiently upregulated during rice flower development with peak matching JA-Ile maximum in the flowers. In both *Osjar1* and *hebiba* mutants, expression was lost due to lack of jasmonate signal. To obtain independent plants with desired graduation of silencing levels (complete knock-outs are expected to be sterile), silencing by RNAi approach was used after selecting gene specific fragments in each target gene. Constructs were introduced

to rice cv. Nipponbare under control of rice ubiquitin promoter using Agrobacterium transformation protocol and pANDA derivative binary vectors. T0 generation plants were successfully regenerated on selective media and 30 lines each were transferred to containment greenhouse for further screening of transgenic plants, flower observations and T1 seed collection. As characterization of transgenic plants and effects of NAC, MADS-box and AP2 genes on flower morphology requires much more time and careful observations, these results will be released gradually after completion of all necessary and sufficiently replicated experiments in the future publications from the group.

(6) Other aspects of jasmonate control and function in rice

Although the role of JA in flowers was the main theme in this project, understanding dual role of JA in development and defense was not less important in our work. In this respect, for instance, we extensively studied the function of JA in control of production of defensive metabolites in rice plants. In particular, we focused on phenolamides that are known to be controlled by wound-induced oxylipins in other plants such as tobacco. Here we found that while these metabolites are indeed controlled by JA-Ile in the leaves, and their levels decreased in *Osjar1* and *hebiba* wounded leaves as expected, their levels were independent of JA signaling in flowers, or even increased in JA-deficient flower parts. Similar pattern was found for diterpene phytoalexins. This is one of the key findings because it shows that defense functions of JA pathway remain restricted to vegetative (leaf) tissues, while during reproduction JAs take over other functions, and thus metabolic regulation is diverted under different and independent control pathways. We are now investigating the switch and molecular mechanisms involved in such differential leaf- and flower-tissue-regulation processes. As integrated part of this research, we also reported novel mechanisms in rice plants to detect an important pest brown planthopper through insect honeydew secretions and JA signaling that involves microbial symbionts of the insect pest. We make progress in characterization of novel insect elicitors, and finally, we bring new insights into JA-controlled volatile emissions from rice during specialist and generalist herbivore attacks.

(7) Overall conclusions and impacts

We characterized in detail hormonal contents in rice flowers, finding that high levels of JA-Ile coincide with anthesis stage in this important monocot model. Secondly, we identified multiple novel targets of JA signaling in flowers, some of which belong to regulatory class genes. Out of them, novel NAC, MADS-box and AP2 genes of yet unknown functions were depicted as potential master regulators of fertility in rice. Furthermore, we made progress in understanding metabolic defense in rice that is controlled by JA in vegetative tissues but becomes uncoupled from JA during reproductive stages. Overall, our research advances the understanding of crosstalk between growth and defense that is important to assure optimal fitness of plants in nature.

５．主な発表論文等 (Research outputs)

〔雑誌論文〕（計 3 件）

〔Main papers〕(3)

① Wari D., Alamgir K.Md., Mujiono K., Hojo H., Shinya T., Tani A., Nakatani H., Galis I. Honeydew-associated microbes elicit defense responses against brown planthopper in rice. Journal of Experimental Botany, 査読有, Vol. 70, 2019, pp. 1683-1696
DOI: 10.1093/jxb/erz041

② Shinya T., Yasuda S., Hyodo K., Tani R., Hojo Y., Fujiwara Y., Hiruma K., Ishizaki T., Fujita Y., Saijo Y., Galis I. Integration of danger peptide signals with HAMP signaling amplifies the anti-herbivore defense responses in rice. The Plant Journal, 査読有, Vol. 94, 2018, pp. 926-637
DOI: 10.1111/tpj.13883

③ Sobhy I.S., Miyake A., Shinya T., Galis I. Oral Secretions affect HIPVs induced by generalist (*Mythimna loreyi*) and specialist (*Parnara guttata*) herbivores in rice. Journal of Chemical Ecology, 査読有, Vol. 43, 2017, pp. 929-943
DOI: 10.1007/s10886-017-0882-4

〔学会発表〕（計 19 件）

〔Conference presentations〕(19)

① Ho N.T., Shinya T., Galis I. Pilot field experiments for identification of novel herbivory-related QTLs by the use of rice BIL population. 60th Annual meeting of JSPP, MAR 13-15, 2019, Nagoya.

② Tohi T., Mujiono K., Shinya T., Galis I. Development of robust method for measurement

of internal leaf volatiles in rice. 60th Annual meeting of JSPP, MAR 13-15, 2019, Nagoya.
③ Shinya T., Fujiwara Y., Hyodo K., Yoshimi Y., Hara K., Yoichi T., Kotake T., Galis I. Analysis of cell wall-derived elicitors during herbivory in rice. 60th Annual meeting of JSPP, MAR 13-15, 2019, Nagoya.
④ Takahashi H., Andama J.B., Hojo Y., Shinya T., Galis I. Two rice homologues of tobacco MYB8 gene do not significantly affect phenolamide levels in herbivory-elicited rice leaves. 60th Annual meeting of JSPP, MAR 13-15, 2019, Nagoya.
⑤ Andama J.B., Shinya T., Galis I. Update on mechanisms of NERICA rice direct defense against insect herbivores. 60th Annual meeting of JSPP, MAR 13-15, 2019, Nagoya.
⑥ Mujiono K., Shinya T., Galis I. The role of jasmonates and ethylene in elicitation of secondary metabolism in rice. 60th Annual meeting of JSPP, MAR 13-15, 2019, Nagoya.
⑦ Galis I., Fukumoto K., Hojo Y., Nakatani H., Shinya T. A hormone with multiple roles: Jasmonate-related functions in defense and development of rice plants. 34th Annual meeting of the International Society of Chemical Ecology, AUG 12-17, 2018, Budapest, Hungary.
⑧ Mujiono K., Sobhy I., Shinya T., Galis I. Jasmonates …but who are “the others” in rice plant defense? 34th Annual meeting of the International Society of Chemical Ecology, AUG 12-17, 2018, Budapest, Hungary.
⑨ Takahashi H., Andama J.B., Hojo Y., Shinya T., Nakatani H., Galis I. Identification of novel genes involved in phenolamide biosynthesis and regulation in rice. 59th Annual meeting of JSPP, MAR 28-30, 2018, Sapporo.
⑩ Shinya T., Hojo Y., Miyamoto K., Uchida K., Yamane H., Okada K., Galis I. OPDA signaling contributes to regulation of phenolamide biosynthesis in rice defense. 59th Annual meeting of JSPP, MAR 28-30, 2018, Sapporo.
⑪ Galis I., Fukumoto F., Hojo Y., Nakatani H., Shinya T. Phytohormone levels and associated gene expression in developing rice panicles. 59th Annual meeting of JSPP, MAR 28-30, 2018, Sapporo.
⑫ Andama J.B., Osinde C., Shinya T., Galis I. Characterization of herbivory resistance traits in NERICA rice varieties. 59th Annual meeting of JSPP, MAR 28-30, 2018, Sapporo.
⑬ Galis I., Osinde C., Tsutsumi N., Iwata H., Kanegae H., Fujimoto M., Takanashi H., Ishimori M., Murage H., Sakamoto W. Assessing the sorghum variability in resistance to insect pests. 58th Annual meeting of JSPP, MAR 16-18, 2017, Kagoshima.
⑭ Galis I., Shinya T., Alamgir K.Md., Sobhy I., Tanabe K., Miyake A., Hojo Y., Osinde C., Sakamoto W., Nakabayashi R., Saito K. Omics everywhere: How about in chemical ecology? 33rd Annual meeting of the International Society for Chemical Ecology and the 9th meeting of the APACE, AUG 23-27, 2017, Kyoto.

〔その他〕
ホームページ等 (Homepage)
http://www.rib.okayama-u.ac.jp/PIN/index.html

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