FELLOWSHIP SUMMARY REPORT

Theme 3: Transformational technologies and innovation

Title: A safe genome editing to develop sporeless mushrooms – for sustainable agriculture without genetic pollution

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I consent to post my Fellowship Summary Report on the Co-operative Research Programme's website.

1. What were the objectives of the research project? Why is the research project important?

Genetic pollution is defined, according to FAO, as the "uncontrolled spread of genetic information (frequently referring to transgenes) into the genomes of organisms in which such genes are not present in nature" and is concerned a risk to ecosystems. Genetic pollution is not limited only to genetically modified or alien species but is also relevant for crops that were artificially mutagenized and selected through breeding processes. Moreover, gene flow between crops, although selected from nature, and their wild relatives affect the evolution of wild populations, possibly resulting in a decrease in genetic diversity and the eventual loss of species during environmental changes such as global warming. Therefore, it can be said that genetic pollution began when people started to practice agriculture. Pausing the genetic pollution caused by agricultural activities is essential for the development of a sustainable society and resilient global ecosystems.

In general, the loss of natural diversity caused by crop cultivation occurs either at the individual level, when seeds, roots, and tubers leak out of the field and invade the ecosystem, or at the genetic level, when gametes such as pollen and spores are transmitted through air or by insects followed by pollination or crosses. The former is relatively easy to prevent and eliminate with proper management, but the latter is largely left uncontrolled. Since the progressive loss of genetic diversity is not immediately apparent, the risk of species loss in the ecosystem is difficult to measure and once lost, genetic diversity is difficult to recover. Currently, the genetic invasion of ecosystems by pollen and spores due to agriculture and forestry is mostly unchecked. In East Asia, researchers of mushroom cultivation have speculated that many of the strains collected from the field are foreign cultivars or derivatives on the analysis of specific phenotypes such as the mating type. In addition to genetic pollution, the scattering of spores and pollen into the environment has negative impacts on human health, such as pollinosis, allergies, and opportunistic infections.

In this project, we aimed to develop a safe genome-editing technology to isolate sporeless oyster mushroom cultivars by combining advanced genetic technology from Kyoto University, KU, Japan with the knowledge and resources of the Public University of Navarra, UPNA, Spain. The aims of this project were as follows:

1. Development of a new safe technology for non-GM genome editing
2. Isolation of sporeless cultivar as a practical example to prevent genetic pollution
3. Experimental check of the absence of heterogenous sequences and unexpected phenotypes during cultivation

2. Were the objectives of the fellowship achieved?

I can say that mostly achieved. Among the three objectives mentioned above, the first two were mostly completed and waiting for publication. While objective 3 has not been achieved yet: genome resequencing of the isolated strains to check the absence of heterogeneous sequences is under progress and phenotypes during cultivation, such as appearance and fragrance of fruiting bodies, have to be done in the future.

3. What were the major achievements of the fellowship? (up to three)

The major achievements for each objective are as follows:

1. Development of a new safe technology for non-GM genome editing
   We have tried to develop safe CRISPR/Cas9 protocols without exogeneous DNA sequence remaining in the bred strains. Among multiple protocols tried, a transnucleous CRISPR/Cas9 protocol employing dikaryotic state with one nucleus as a donor and the other as a recipient for providing Cas9/gRNA complex resulted exogeneous-DNA-free genome-editing in the recipient nucleus. Monokaryotic isolates harboring the genome-edited recipient nucleus were able to be obtained after dedikaryotization through protoplast regeneration.
2. Isolation of sporeless cultivar as a practical example to prevent genetic pollution
   Several new genes essential for spore formation were found through gene knock-out experiment with CRISPR/Cas9 to screen for sporeless phenotype among disruptants of candidate genes nominated from comparative transcriptomic analyses between different specimen of the fruiting body. These knock-out strains were demonstrated not to form spore by being arrested at a step after meiosis.
3. Experimental check of the absence of heterogenous sequences and phenotypes during cultivation
   Using PCR and Southern blot, we demonstrated absence of exogenous DNA remaining in the genome of the isolates. Genome-resequencing of the isolates to check if there are small piece of exogenous DNA

sequence or unexpected rearrangements are now under way. The shape of fruiting body was checked and seems normal in preliminary experiments. However detailed characterization of yield, quality and post-harvest properties should be examined in the future.

4. Will there be any follow-up work?

After finalizing research experiments, several papers will be published in an international scientific journal with high standard levels. Furthermore, we have co-authorized a mini-review and submitted it to a Q1 journal, Applied Microbiology and Biotechnology. This manuscript is now under the review process and will be published in the early 2024 after acceptance.

Moreover, beside the specific topic on breeding a sporeless strain, we started another collaboration project combining our genome editing technology and bioinformatic analysis in host laboratory. To do this more active and continuously, we submitted a proposal for international exchange program supported by Japanese government. We are looking forward to future new collaboration and exchange of researchers and students on the new topics. On the intellectual properties, we have concentrated to do basic research and do not have any concrete topics for patent applications for the moment.

5. How might the results of your research project be important for helping develop regional, national or international agro-food, fisheries or forestry policies and, or practices, or be beneficial for society?

The isolation of a safe non-GM genome-edited cultivar that is beneficial to both humans and the environment will have a significant impact on stakeholders, including policy makers, and lead them to consider the regulation of genome-edited crops. Until now, regulations on genome editing vary among the countries: genome-edited crops are treated as non-GM in USA or Japan, if no transgene sequence is left, but CRISPR plants are subjected to GM laws in the EU. Moreover, it has been reported that the UK is considering loosening rules for genome-edited crops and animals after Brexit. Thus, it is very important to consider the utilization of genome-edited crops with evidence and a scientific assessment of the associated advantages and risks.

In addition, the successful isolation of a sporeless cultivar will call attention to the risk of genetic pollution caused by agricultural activities. In addition to mushroom spores, other gametes, such as pollen, have a severe impact on genetic diversity in natural populations as well as on human health in some cases. The strain developed in this project will be a solution to this environmental problem and to promote sustainable agriculture by reducing pressure on the environment.

6. How was this research relevant to the objective and research theme of the CRP:

The obtained mushroom strains can serve as a pilot model for non-GM genome-edited cultivars in other crops. They practically contribute to the 'SUSTAINABILITY' goal by developing an ecofriendly technique for use in agriculture that is safe for humans and the environment without the distribution of the spores and pollen. The results also have much impact on the 'FOOD SECURITY AND NUTRITION' goal by validating a safe and efficient non-GM CRISPR/Cas9 protocol that can lead the effective utilization of genome editing for production of food and feed required for the growing global population.

As for the three themes of the CRP, our project directly contributes to Theme III, 'TRANSFORMTIONAL TECHNOLOGIES AND INNOVATION' as it provides a practical and fundamental protocol for non-GM genome editing in mushrooms that will also be applicable to other crops. In addition, it has a significant impact on Theme I, 'MANAGING NATURAL CAPITAL', as this project aims to provide a solution for genetic pollution caused by agriculture and thus, to maintain genetic and species diversity in natural bioresources. Furthermore, this project can also contribute to Theme II, 'STRENGTHNING RESILIENCE IN THE FACE OF MULTIPLE RISKS IN A CONNETED WORLD' by preventing transboundary genetic pollution to maintain the integrity of regional bioresources and by halting the loss of resilience against various environmental risks caused by climate change.

7. Satisfaction

I am totally satisfied with the OECD CRP fellowship to support me during the stay in Spain to do collaboration and strengthen network between Spain and Japan.

I did not encounter any practical problems. Actually, it was thankful that OECD permitted me to postpone the visiting schedule by one year because of the uncertain situations during the COVID pandemics. And to my luck, the payments were in EUR that protected me against the rapid change in the currency rate between EUR and JPY. For someone who may visit other countries outside of EU, this kind of change in currency exchange rate could be a problem in the CRP.

8. Advertising the Co-operative Research Programme

I came to know about the CRP through home institution mailing list and I attended an online seminar held by the contact persons in the Ministry of Agriculture, Forestry and Fishery, Japan. I may suggest active utilization of SNS tools or mailing list of researchers who are interested in OECD or FAO activities. I do hope that many colleagues may use this precious opportunity to make solid relationship with people abroad to contribute a sustainable future.

I wish to thank OECD, particularly to Mrs. Nathalie Elisseou for the efficient administration of the documentation and payments. I am also grateful to Drs. Ichiro Nakayama and Yuji Yasukochi who gave me useful instruction and advice upon application. Last but not least, I would like to appreciate so much all the people in the host laboratory, especially, Profs. Antonio G. Pisabarro and Lucía Ramírez, for their warm and welcoming hospitality, as well as my colleagues in Kyoto University for accepting my six-months absence.