FELLOWSHIP SUMMARY REPORTS

Cover page

- Francisco Barro Losada
- Theme III.TRANSFORMATIONAL TECHNOLOGIES AND INNOVATION
- Host institution: Center for Precision Plant Genomics, University of Minnesota (USA)
- Host collaborator: Daniel F. Voytas
- The dates of fellowship: July 11 – December 21, 2022
- I consent to this report being posted on the Co-operative Research Programme's website

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

The use of gene-editing techniques such as CRISPR/Cas has been shown to be very efficient for introducing point mutations. The stay aimed to update knowledge of CRISPR/Cas-directed mutagenesis technologies and their specific application in a research project that aims the redesigning the composition of the hexaploid cereal grain (oats) by providing it with unique baking characteristics, which it currently lacks. The stay covered the following specific objectives:

1. Genetic analysis and NGS sequencing data for the selection of target regions for CRISPR/Cas.
2. Design of sgRNAs for gene editing and construction of CRISPR vectors containing also a GFP marker gene to monitor the efficiency of transformation and gene editing.
3. Proof-of-concept testing of selected designs in an *in vitro* cereal protoplast system; sequencing of target regions, data analysis, and selection and construction of final vectors.

Oat is a cereal with nutritional properties superior to those of wheat, but it cannot be used in baking because its proteins do not form a protein network that gives the dough the elasticity and extensibility characteristic of bread wheat. However, it is a cereal that can be consumed by coeliacs, those sensitive to gluten and other pathologies such as irritable bowel syndrome, and those on a low FODMAPs diet. Achieving this objective would make oats a baking cereal, a cereal with high added value, not only for the gluten-intolerant community but also for the general population, farmers, and other stakeholders of the cereal value chain.

2. Were the objectives of the fellowship achieved?

Yes, the objectives proposed for the stay are mainly achieved, although we are still working on objective 3 as sequencing data analysis is time-consuming and results are not yet ready.

In the first objective, oat sequencing data from grain protein genes were analysed. Data were obtained from two Spanish genotypes and two additional genotypes from the University of Minnesota. This information is valuable for designing the sgRNAs necessary to knock out oat storage protein genes. Several highly conserved gene regions were identified for all four oat genotypes.

In the second objective, six sgRNAs guides were designed and two different types of editing vectors were constructed. The first type uses a U6 promoter (RNA polymerase III promoter) driving the expression of two sgRNA guides, and therefore, three different vectors were constructed to arrange all six sgRNAs. In all three expression vectors, the GFP gene was included to evaluate the transformation efficiency in protoplasts or later for monitoring stable transformation during *in vitro* regeneration process to obtain the final edited plants. The second type of vector was assembled by using a gRNA

spacer array for tRNA into MoClo-compatible vectors, which allows to the assembly of up to 6 sgRNAs for SpCas9 with any RNA-pol II promoter or terminator. Next, the array of six sgRNAs was assembled into intermediate vectors under the control of promoters from the *Panicum virgatum* polyubiquitin gene (PvUbi1) or the Cestrum yellow leaf curling virus (CmYLCV). Later, these intermediate constructs were assembled into the final constructs using a Golden Gate reaction using modules containing the SpCas9, GFP, and hygromycin as the selective agent. All vectors were used for protoplast transformation and brought to the lab in Spain for generating stable edited oat plants.

In the third objective, all constructs were tested in an oat protoplasts system. To that, protoplast isolation and transformation protocol from oat was established. The cell wall was digested using a mixture of enzymes (cellulase R10 and macerozyme R10) in an MES buffer. A PEG-mediated transformation system was used for protoplast transformation. In this protocol, variables like plant age, protoplast transformation density, temperature treatment, vector concentration, incubation time after transformation, etc., were tested. The expression of GFP was used as an indicator of the transformation efficiency. As an outcome, the conditions for oat protoplast were set and used for further transformation experiments which included the final vectors containing the sgRNAs. In total, nineteen different experiments were to set up the transformation system and the efficiency of the sgRNAs-containing vectors. Finally, DNA from protoplasts was isolated and used for PCR amplification of target regions, and sent for DNA sequencing. However, DNA sequencing results showed multiple contaminations from non-target DNA, and particularly from species not related and very distant from Oat. Currently, we are in conversations with the company that carried out the sequencing as we suspect that contamination comes from their side. They have resequenced some samples changing sequencing conditions that notably improved sequencing results. Now, they will apply these new conditions for resequencing the rest of the samples.

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

The three main achievements of the fellowship are:

1. Strengthened collaboration with the host laboratory, which has allowed us to initiate new challenges and propose them as new joint research projects.
2. A robust protocol for protoplast isolation and transformation from oat leaves was established.
3. Ten different vectors containing sgRNAs to target oat grain protein genes were developed and tested in protoplasts. These constructs are expected to be used in the Spanish laboratory for the stable transformation and gene editing in oat.

4. Will there be any follow-up work?

As an outcome of the fellowship, it is expected one publication in Q1 scientific journal describing the methodology and use of oat protoplasts for gene editing and redesigning protein balance in the oat grain. Currently, we are waiting to solve the sequencing issues described previously to include them in the manuscript. Results also provided the basis to strengthen the collaboration between the Spanish and host laboratory, which allow us to address new joint research projects.

As mentioned before, the final goal of the project is redesigning the protein distribution in oat to make it more amenable for breadmaking. Results from oat protoplasts are preliminary, and we need to develop stable edited oat plants to find out success. If so, those results could be protected or provided the new innovative raw material for gluten-free products.

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 results of this project will have an important impact on the regional, national, or international agro-food system. On one hand, diseases related to gluten/wheat ingestion have greatly increased in recent years in Western countries, becoming a real public health problem. Unlike other diseases, people suffering from coeliac disease or other gluten-related disorders do not base their treatment on medication but on a lifelong Gluten-Free Diet (GFD). Because flour used for GFD does have not the breadmaking properties of wheat, several additives are added that strongly influence increasing glycemic index as well as other health parameters and deteriorating gut microbiota. Oat is safe for the treatment of gluten-related disorders but with limited breadmaking properties. Results from this project will provide an excellent raw material for the treatment of these pathologies.

Finally, the results of this project can also be used to help policymakers, as there are currently very few projects demonstrating the usefulness of gene-editing techniques to improve the quality of life of citizens. The results of this visit/project go beyond the scientific aspect, they can be an excellent tool for legislators to influence national or international legislation on gene editing. It is a clear example of the inclusion of minorities across the OECD, such as those with gluten intolerances. It is also a clear example of generating valorization of existing crops or offering new alternatives to farmers.

6. How was this research relevant to:

- The objectives of the CRP?
- The CRP research theme?

The key outcomes of the CRP objectives are to improve sustainability, food security, and nutrition. These are also the main challenges that agriculture systems are facing in a climate change context to satisfy food demand from an increasing population. New crop varieties and more resilience to climate change have to be developed in order to maintain or increase the resilience of the food system as well. This research is relevant and in line with those principles. We are applying precision genome editing, and biotechnology tools as new significant opportunities to enhance crop breeding to address agricultural productivity constraints, and issues related to food security, human nutrition, and health. The new oat varieties with improved breadmaking qualities, that will be developed in this project using CRISPR/Cas technology, are in line with Theme III of the CRP themes. This project also considers minorities whose nutritional requirement are often relegated and few times are the target for new developments. As a consequence, this project not only has a scientific dimension but also social and economic ones, to improve the quality of life of people suffering from gluten-related disorders.

7. Satisfaction

- Did your fellowship conform to your expectations?
- Will the OECD Co-operative Research Programme fellowship increase directly or indirectly your career opportunities? Please specify.
- Did you encounter any practical problems?
- Please suggest any improvements in the Fellowship Programme.

This is the first time I had an OECD fellowship but previously I had other fellowships to carry out short scientific stays. I have to say that the OECD fellowship greatly confirmed my expectation, particularly in two important aspects; Scientific and Management.

From the scientific point of view, the OECD Co-operative Research Programme fellowship allowed me to increase my career opportunities; by improving the collaboration with the host laboratory, by exchanging new innovative ideas, and by proposing cutting-edge approaches to achieve the final objective of the project. This collaboration is now beyond the stay, we have joined project proposals, and student exchange. In addition, during my stay, I had the opportunity of attending high-quality seminars and meetings, which allowed me to update the genome editing technology. The host laboratory is one of the most competitive in terms of gene-editing reagents, and this was also very valuable to implement such technologies in my project.

On the other hand, the management was well established, payments were as scheduled and on time. In summary, my satisfaction with the OECD CRP fellowship is high as compared with others.

Regarding issues or practical problems. As mentioned previously, no scientific or management problems were found with the OECD fellowship. However, I have to mention two issues in which I had practical problems: the first was the accommodation, as it was very complicated to find out suitable accommodation at a reasonable price; and the second was the current inflationary global context, which is also affecting the US. This context has caused prices to be really high, making the fellowship allowance insufficient for the full stay. Therefore, my recommendation is to consider an update of the fellowship considering the inflation variable,

8. Advertising the Co-operative Research Programme

- How did you learn about the Co-operative Research Programme?
- What would you suggest to make it more "visible"?
- Are there any issues you would like to record?

I learned about the OECD fellowship through colleagues from my institute in Spain. They also recommended applying for me. They highlighted the efficiency and the lack of bureaucracy, in contrast to other fellowships. I have to say that this is really true, and this is really important because, for these short stays, the most important is the stay and not to deal with complicated and time-consuming paperwork.

I strongly recommend this fellowship to other colleagues. However, it is also true that not many researchers are aware of it. To increase the visibility of the calls, it could an excellent idea to contact the communication department of institutions like CSIC in Spain, INRAE in France, and others, which are widespread all over the country. On the other hand, Twitter and Instagram are increasing among research institutions and researchers.

Córdoba, February 5, 2023

Professor Francisco Barro Losada

Institute for Sustainable Agriculture
Spanish National Research Council