Investigating the genetic processes that support Ensifer Mediated Transformation

Key external stakeholders:
Ag-biotech sector, public research and breeding organisations and seed breeding companies

Practical implications for stakeholders:
Ensifer-Mediated Transformation (EMT) is a novel gene transfer technology platform that facilitates the engineering of crop genomes in order to for example enhance the agronomic potential of a variety (drought/disease tolerance) and/or to enhance end-user traits such as oil quality or flavor. Based on external analysis completed by ag-biotech consultants, EMT has the potential to be a technology that could be licensed broadly across the agbiotechnology and seed breeding sectors on a non-exclusive crop-by-crop basis. Globally, it has been estimated that there are between 40 and 70 potential commercial licensees for EMT. This project was developed to expand the potential of EMT by completing fundamental research studies into the activity and structure of the Ensiler adhaerens genome, while investigating at an applied level the capability of EMT to deliver gene editing components into plant cells and to determine the potential for EMT to effectively transform wheat.

The outcome/technology or information/recommendation is that:
- The genome sequence of E. adhaerens has been fully characterised, with the resulting datasets providing an essential research resource for all future EMT work.
- The genetic processes within E. adhaerens that support EMT have been fully identified by studying the activity of all genes within the E. adhaerens genome during EMT
- EMT can successfully deliver the components for gene editing into plant cells and provides a novel platform for gene editing in plants
- Wheat is not amenable to EMT with current protocols

Main results:
- The structure and content of the E. adhaerens genome has been fully characterised and annotated.
- Gene activity within E. adhaerens has been detailed through the EMT process, which has led to the identification of multiple genes which are intrinsic for supporting EMT through its core stages.
- Unlike other dicot and monocot crop species, wheat is not amenable to EMT using current protocols.
- Primary components for gene editing can be delivered into plant cells using EMT.

Opportunity / Benefit:
Output from this project has provided a series of fundamental datasets, which provide key insights into the workings of E. adhaerens during EMT while also detailing the genetic structure of the bacterium relative to other plant transforming bacteria. In addition, this project highlights the potential for EMT to facilitate gene editing for future plant enhancement strategies.

Collaborating Institutions:
UCD

Contact
Ewen Mullins
Email: ewen.mullins@teagasc.ie.
http://www.teagasc.ie/publications/
Teagasc project team: Evelyn Zuniga-Soto, Steven Rudder
Dr. Ewen Mullins (PI)
External collaborators: Prof. Fiona Doohan, UCD

1. Project background:
The project idea centres on addressing significant gaps in our basic understanding of the genetic processes within *E. adhaerens* that support the gene transfer process that is Ensifer Mediated Transformation (EMT). Complementary to this, the project was tasked with investigating the potential utility of EMT for accommodating current tools for gene editing while also examining the potential application of EMT to wheat, which is considered one of the more difficult crop species to transform.

The target market for EMT is primarily the biotech/GM seed market which globally is worth ~$14.5 billion. Although agrochemicals represent a larger market share at present, with a global market value of approximately $46 billion in 2009, the growing trend over the past two decades has been a shift away from the development of new agrochemical molecules towards the use of biotechnology. China, India, Brazil, Argentina and South Africa are the 5 leading developing countries involved with biotech crops, and collectively grew ~44% of the global market share.

The overarching goal of the study was to enhance the commercial potential of EMT, a novel gene transfer system which can be used to improve the agronomic performance and/or enhance the end-user value of major crop species. EMT technology is equivalent to current gene transfer platforms that are dependent on the market alternative, Agrobacterium-mediated transformation (‘AMT’). Critically, EMT bypasses several of the restrictive patents (controlled by multi-national ag-biotech companies) that currently impede users of AMT from developing commercially viable products. To date, the potential of EMT has been demonstrated on potato, tobacco, rice, oliseseed rape and on cassava, a primary stable food crop of Africa and Asia. While the technical proficiency of EMT has been demonstrated on several crop species, the underlying genetic systems that support EMT are unknown. Elucidating these processes is key to enhancing the utility of the technology platform and supporting the current Go-To-Market strategy for exploiting EMT, which has been previously compiled by independent consultants (see Project 6603).

2. Questions addressed by the project:
- What is the genome structure and sequence of *Ensifer adhaerens*?
- What are the most relevant gene networks activated within the *E. adhaerens* genome during EMT?
- Can EMT genetically engineer wheat using current protocols?
- Can EMT facilitate precision breeding through the transfer of gene editing components into plants?

3. The experimental studies:
The OV14 genome was sequenced and constructed by BaseClear B. V. Leiden, Netherlands. A hybrid approach using the Illumina HiSeq and PacBio RS platforms was selected. The genome was constructed from 1GB Illumina paired-end reads, 500MB Illumina mate paired end reads, and 100MB PacBio RS reads. The genome sequence is available in the NCBI database under accession numbers CP007236 (scaffold 1), CP007237 (scaffold 2), CP007238 (scaffold 3) and CP007239 (scaffold 4). Post-sequencing the resulting genome of *E. adhaerens* was compared against previously published sequences of two other plant transforming bacteria, *Agrobacterium tumefaciens* (which supports AMT) and *Sinorhizobium meliloti*, which supports SMT. To study total gene activity within the genome of *E. adhaerens* during EMT, the transcriptome of *E. adhaerens* was characterized by extracting total RNA from *E. adhaerens* cells that were co-cultivated with plant roots for periods up to 7 days. RNA extraction was achieved using the Trizol-based procedure. Subsequent, rRNA depletion, cDNA library preparation and RNA sequencing of each sample was performed under contract by the Beijing Genomics Institute (BGI). Paired end sequencing was completed on a HiSeq 4000 System (TruSeq SBS KIT-HS. Illumina), with an expected read length of 100 bp. Depth coverage was calculated at 10-12 million reads per sample. Separately, wheat transformations were attempted on both mature and immature embryos of cv. Bobwhite using previously published methods (and variants of) for AMT (Ding et al. 1999; Jones et al. 2004). Variations of the methods included modifications to the bacterium-plant co-cultivation period and the concentrations of the selection agent (hygromycin) during the subsequent tissue culture steps. For gene editing, single guide RNAs targeting the GUS marker gene in Arabidopsis were designed and cloned into a CRISPR/Cas9 containing plasmid according to Li et al (2015). The pUC-119-gRNA plasmid was used as a template to amplify the A. thaliana ubiquitin promoter (AtU6-1).
and the corresponding gRNA scaffold. The pFGC-pcoCas9 plasmid was selected because it contains a plant-optimized version of the cas9 gene (Li et al. 2013; Li, Zhang, and Sheen 2015). Correct assembly of the sgRNAs within the pGEMT-Easy and then the pFGC-pcoCas9 plasmid was confirmed by Sanger sequencing (Macrogen, Seoul, South Korea). The final plasmid named as pFGC-pcoCas9-GUS(1-2)-Gent (Figure 5.1) was then transformed into E. adhaerens OV14_p5105 (EOV14_5105) by electroporation.

4. Main results:

Of the three plant transforming bacteria tested the E. adhaerens genome is the largest at 7.71Mb, which is 2.04Mb bigger than the A. tumefaciens genome and 1.03Mb larger than the S. meliloti genome. The E. adhaerens genome is composed of four structural units; chromosome 1 and 2 and two smaller plasmids, plasmid B and C. Of interest is the fact that E. adhaerens and A. tumefaciens share a similar sized mobile plasmid of ~150kb in size which is not found in S. meliloti; the least efficient bacterium for plant transformation. Examining the level of identity across all three genomes it was clear that there was a higher degree of relatedness (i.e. synteny) between the whole genome of E. adhaerens and S. meliloti than that of E. adhaerens and A. tumefaciens (Figure 1). Indeed phylogenetic analysis of all three indicate that E. adhaerens is more closely related to S. meliloti than it is to A. tumefaciens, highlighting the genetic distinctness between E. adhaerens and A. tumefaciens.

![Figure 1: Comparative synten plots of Agrobacterium tumefaciens (C58: top bar), Ensifer adhaerens (OV14: middle bar), and Sinorhizobium meliloti (1021: bottom bar). The replicons within each genome are separated by coloured bars, with synteny displayed via red lines representing direct homology with blue lines corresponding to inverted homology.](image-url)

Overall, no genes homologous to the A. tumefaciens Ti plasmid vir genes (which drive the transformation process) were present in the genome of E. adhaerens, which confirms the need to augment E. adhaerens with additional vir genes to facilitate EMT. This is achieved with the addition of the pC5105 plasmid in all EMT applications. Of significance, genes that are non-essential but exert a positive influence on virulence and the ability to genetically transform host genomes were identified in E. adhaerens, indicating that while it may be a symbiote, E. adhaerens retains the ability to transform plant cells (when augmented with pC5105) because it contains multiple genes that also support the ability of A. tumefaciens to transform cells.

Investigating total gene activity within the E. adhaerens genome during EMT, optimum transformation of plant tissues was achieved ~7 days after inoculation of the host with the bacterium, with the number of identified genes that were differentially expressed (DEGs) increasing substantially after longer periods of cocultivation with plant tissue (Figure 2). Across the timepoints (D0, D1, D3, D5, D7) studied, 52,877 E. adhaerens DEGs were expressed following exposure to plant tissue versus 34,287 in the absence of plant material. A major 9.3-fold increase in DEGs was found in the transition from D0 to D1, compared to values ranging from 1.5- to 2.5-fold from D2 to D7. The processes of biofilm formation, flagellar assembly and quorum sensing are closely intertwined and related to successful plant transformation with AMT. The presence of functional flagella on E. adhaerens has been previously confirmed and a suite of DEGs involved in flagella structural assembly and motor responses were identified indicating their important role in the initial perception of plant tissues by E. adhaerens.
Figure 2. Number of genes up-regulated (red) and down-regulated (green) for each of the 4 replicons (chr1, chr2, pb, pc) within E. adhaerens, plus the additional plasmid (pS105).

Based on current EMT protocols, E. adhaerens OV14 can accommodate the machinery necessary for CRISPR-Cas9 editing. In this study, the % of GUS foci within the treated Arabidopsis roots, decreased significantly after 3 days co-cultivation in the presence of the CRISPR plasmid. Molecular analysis confirmed the successful transfer of the CRISPR/Cas9 components into the treated plant tissues.

Figure 3. Impact of CRISPR/Cas9 editing as facilitated by EMT with reductions in %GUS foci staining compared to standard EMT control

In regards to the application of EMT for wheat transformations, the infection efficiency of wheat immature embryos by E. adhaerens was increased, however further work is required to develop the regeneration protocol in order to achieve > 90% of embryogenic calli and shoot regeneration.

5. Opportunity/Benefit:
The work completed in this project has generated fundamental datasets covering the genetic structure and activity of E. adhaerens during EMT. This data has led to the identification of gene(s) families, whose manipulation should enhance the efficiency of EMT in future work. While EMT is not compatible with wheat transformation at present, the ability of EMT to deliver gene editing expands the potential application of the technology further. Combined the output from this project provides the EMT programme, and Teagasc, with the opportunity to enhance the operability of EMT and licensing potential for Teagasc.

6. Dissemination:
Scientific output was disseminated at international scientific conferences including:
- Crown Gall Conference on Genetic Engineering, Indianapolis, Indiana, November 12th – 16th, 2015

Main publications:
Rudder et al. (2014). Genome sequence of Ensifer adhaerens OV14 provides insights into its ability as a novel vector for the genetic transformation of plant genomes. BMC Genomics, 15. 268.

Compiled by: Dr. Ewen Mullins