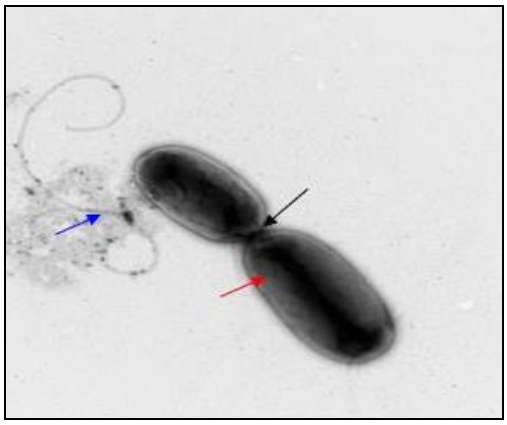
Project number: 6575
Funding source: Teagasc
Date: Dec 2019
Project dates: Oct 2015 – Nov 2019

Expanding the utility of Ensifer-Mediated Transformation: a novel technology platform to engineer crop genomes



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 technology platform that facilitates the engineering of crop genomes in order to enhance the agronomic potential of a variety (drought/disease tolerance) and/or to enhance end-user traits. Based on external analysis completed by ag-biotech consultants, EMT has broad licensing potential across the ag-biotechnology and seed breeding sectors on a non-exclusive crop-by-crop basis. Globally, there are between 40 and 70 potential commercial licensees for EMT. This project was developed to further expand the commercial potential of EMT by (i) characterizing the defence response of a plant following treatment with *Ensifer adhaerens* and (ii) investigating the propensity of *E. adhaerens* to transform a broad range of potato varieties.

The outcome/technology or information/recommendation is that:

- The defence response of a plant to *E. adhaerens* has been fully characterized with datasets highlighting the potential of *E. adhaerens* to reduce the immune response of plants, in contrast to what occurs following treatment with *A. tumefaciens.*
- EMT can be developed into a 'stealth' engineering platform, with the ability to modify plant genomes without eliciting a strong defence response from the plant host. This presents a significant market advantage over currently available engineering systems.

Main results:

- The early responses of plant cells to EMT has been fully characterised, which has elucidated the genetic mechanisms of how *E. adhaerens* interacts with its host.
- Plants treated with EMT do not mount a strong defence reaction to treatment with *E. adhaerens*
- *E. adhaerens* successfully transformed all 10 potato varieties tested in this study, in contrast to *A. tumefaciens,* which is disadvantaged by this high level of genotype dependency.

Opportunity / Benefit:

Output from this project has provided a series of fundamental and applied datasets, which provide key insights into the workings of *E. adhaerens* during EMT while also detailing the genetic response of plants to EMT. This project addresses two of the significant questions being asked by ag-tech stakeholders in regards to EMT: does EMT have a lower level of genotype dependency compared to AMT and what are the genetic mechanisms supporting this phenomenon.

Collaborating Institutions:

UCD

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1. Project background:

Over the past three decades Agrobacterium mediated transformation (AMT) has been shown to have technical limitations due to poor plant tissue regeneration plus recalcitrant responses like browning and necrosis of plant tissues to infection leading to genotype dependency of existing AMT methods. In essence, this typically restricts the application of AMT to a small number of varieties for each crop species, which complicates downstream trait development in elite varieties. Ensifer mediated transformation (EMT) technology is an alternative platform for transforming different crop species using Ensifer adhaerens Ov14. In order to understand and manipulate the EMT process, it is necessary to study how plants respond to E. adhaerens, especially during the early stages of the bacterium-plant interaction and the compatibility of EMT to transform different varieties of a major crop with less genotype dependency. The project idea here centres on addressing the significant gaps in our basic understanding of the genetic processes that occur between *E. adhaerens* and its host plant material that support EMT.

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 by addressing specific queries made to Teagasc by stakeholders in the ag-biotech sector that have expressed interest in licensing EMT for their own trait development pipelines. To date, the potential of EMT has been demonstrated on potato, tobacco, rice, oilseed 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 response of a treated plant remains unknown. This has implications for future licensees in regards to complying with regulatory requirements for the marketing of any GM varieties developed. In addition, the ability of EMT to overcome genotype dependency has yet to be determined. Genotype dependency is a serious limitation with the use of AMT, with the industry currently reliant on time consuming 'work-around' systems to minimize the impact of this phenomenon.

2. Questions addressed by the project:

- What is the genetic response of plants to exposure to *Ensifer adhaerens*
- Relative to AMT, what type of plant defense responses are elicited by EMT
- Can EMT genetically engineer a broader number of varieties in a major food crop

3. The experimental studies:

Gene activity within a treated plant following EMT was recorded using RNA sequencing (RNAseq) of treated *Arabidopsis* roots. RNAseq is superior in both the discovery and the reliable quantification of gene expression and is widely used to examine total gene activity in an organism in response to a specific event/stress. For this study, Arabdiopsis roots were treated with *E. adhaerens* or *A. tumefaciens* and plant tissue samples collected 3hr, 6hr and 12hr post-treatment. Thereafter, RNA was extracted as per standard protocols. After quantification, for concentration and quality (RIN value) check, the Illumina TruSeq stranded mRNA library preparation kit (Illumina, Inc. San Diego, CA, USA) was used to prepare sequencing libraries from mRNA to get a clear and complete view of the transcriptome with precise strand information. After library preparation the samples were run on an Illumina HiSeq 2500 sequencer (Illumina, Inc. San Diego, CA, USA). Following Illumina sequencing, reads obtained were pre-processed before mapping to the Arabidopsis genome. Using the developed RNAseq transcript files comparisons were then made between treated samples versus respective control (e.g. EMT/AMT v. water control) for each respective time-point.

To examine the propensity for genotype dependency with the use of EMT, tissue culture plantlets of 10 potato varieties were generated by excising ~3 cm long sprouts from potato tubers, which were then surface sterilized with 70 % ethanol for 2 min and treatment with 10 % bleach containing 0.1 % tween-20 for 10 min.

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The sterile sprouts were washed 5 – 6 times with sterile water and air dried before being cultured on propagation medium in steri-vent high containers at 24 ˚C under a 16 h/8 h light/dark cycle. For the transient transformation procedure, a standard protocol was applied using a collection of sterile explants from each variety based on a previous protocol developed at Oak Park.

4. Main results:

EMT treatment induced the upregulation of 206 and down-regulation of 225 genes across the Arabidopsis genome. EMT showed almost an equal number of up and regulated gene induction, but at 12hpi there was twice more downregulated genes (n = 133) compared to upregulated genes (n = 49) compared to other time-points (3 and 6 hpi). In contrast, AMT led to the up-regulation of 893 and down regulation of 1373 genes. Of the total number of Arabidopsis genes induced by AMT, approximately 60% were downregulated (n = 1373). The maximum number of genes induced following AMT treatment was observed at 6hpi (n = 1577), as compared to other time-points of co-cultivation (Figure 1). A total of 153 upregulated and 207 downregulated expressed genes were common to both EMT and AMT across all three time-points (3, 6 and 12hpi).

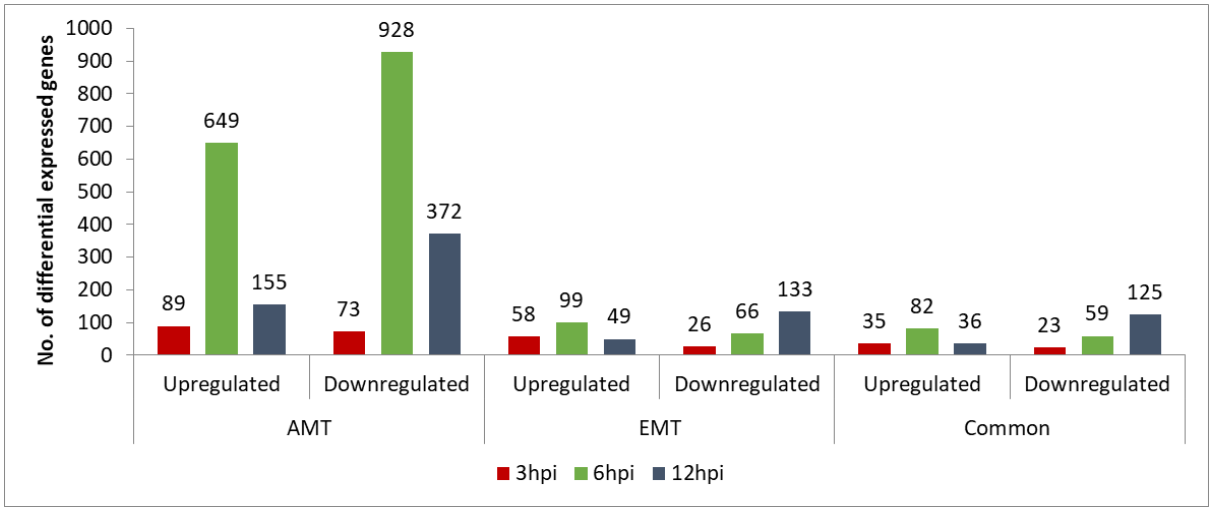

Figure 1. Number of unique genes differentially expressed (either up/down-regulated) in Arabidopsis following treatment with AMT or EMT and genes that were found to be activated in common to both treatments.

EMT upregulated genes were primarily associated with root morphogenesis and development, response to osmotic stress and peroxidase activity, with EMT downregulated genes involved in auxin stimulus, transmembrane transport and anthocyanin biosynthesis process. The time-point of higher gene expression changes observed for AMT was at 6hpi with upregulated genes mostly involved in defense response (systemic acquired resistance), response to reactive oxygen species (ROS), transmembrane receptor kinase pathway, regulation of hormone level and catalytic activity. The AMT downregulated genes were involved in processes like defense against bacteria, hormone signaling (Salicylic acid (SA), Ethylene (ET), Jasmonic acid (JA), Abscisic acid (ABA), and Auxin), MAPK mediated signaling, Immune response (ROS, programmed cell death (PCD), Hypersensitive response (HR)) and membrane protein targeting.

A parallel bioinformatic assessment confirmed that AMT induced more stress/defense responsive genes in Arabidopsis than EMT. LRR-RLK genes are important defense-associated genes in plants, coordinating the transcription of gene groups that collectively support a plant's response to a biotic stress. Out of the 94 Arabidopsis LRR-RLK family genes only 22 genes were significantly induced in this study, irrespective of treatment applied (EMT/AMT). Of this number, AMT significantly induced all 22 LRR-RLK genes identified but EMT only induced 5 LRR-RLK genes.

Successful transformation requires a compatible interaction between the host plant and the transforming bacteria. Exposure of plant tissues to AMT has identified incompatible responses like tissue browning/necrosis and recalcitrance of crops to transformation. This genotype dependency due to tissue browning and necrosis responses to AMT has been reported in multiple crops including potato. In this study each of the ten commercial varieties recorded effective transient transformation efficiencies by EMT compared to AMT, with no genotype dependency observed with EMT (Figure 2). In contrast AMT was only

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capable of transiently transforming Homeguard, Orla, British Queen, Bikini, Desiree and Sarpo Mira.

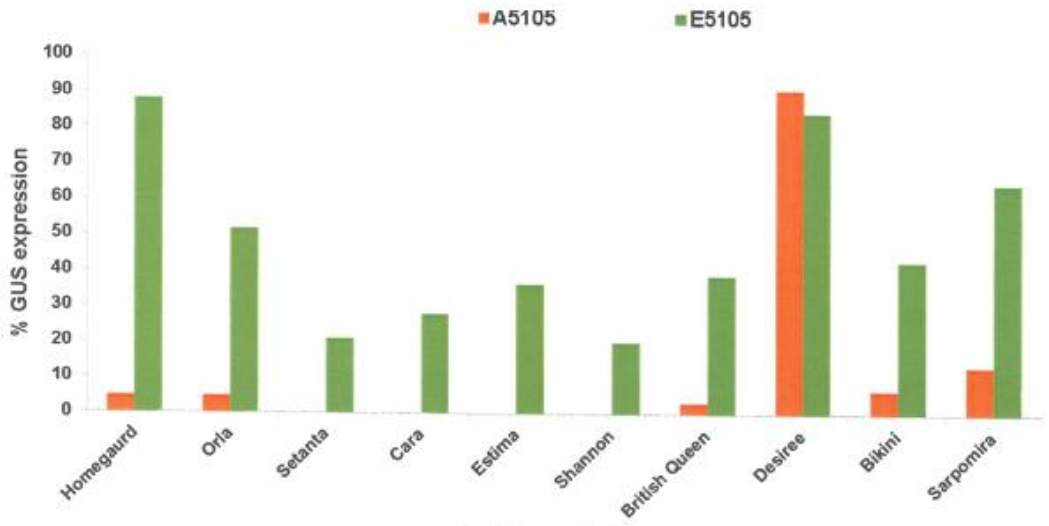

Figure 2. % transient transformation of potato explants across 10 commercial potato varieties following treatment with AMT (A5105) or EMT (E5105). % transformation was recorded based on the presence/absence of GUS staining on treated potato explants.

5. Opportunity/Benefit:

The work completed in this project has generated both strategic and applied datasets of direct relevance to stakeholders in the ag-biotech industry that have expressed an interest in licensing EMT for their commercial trait development pipelines. The novel data generated here provides an objective perspective on how *E. adhaerens* and its host interact during EMT. By identifying the lack of a strong defense response in plants during EMT (versus AMT), the stealth capability of *E. adhaerens* has been underlined. This will support future applications to regulators who will seek information on the genetic response of host material during the EMT process. In addition, the propensity for genotype independency with EMT (for potato) further highlights the utility of EMT against current commercial plant engineering platforms. 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 via invited/conference presentations at the following events:

- Crown Gall Conference on Genetic Engineering, Indianapolis, Indiana, November, 2015
- International Symposium on Plant Transformation Biotechnologies, Taiwan, October, 2016
- International Association of Plant Biotechnology, Dublin, August, 2018

Main publications:

Govinidan, A. (in prep.). PhD Thesis, University College Dublin

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