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Meeting Report: GARNet Gene Editing Workshop: University of Bristol, March 26th-27th 2018.

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Introduction

Gene editing (GE) technology is now widely used by plant scientists who work in a range of model and crop species. GARNet, The New Phytologist Trust and the Bristol Centre for Agricultural Innovation brought a group of global experts to the University of Bristol in March 2018 to discuss current applications, emerging best practice and policy issues that surround use of this technology.

Global legislation and gene editing in plants

Difficult times in Europe

One aim of the workshop was to discuss the regulatory and political issues that surround the use of GE. To this end, invited speakers explained developments from the regions of greatest relevance to delegates, namely the UK, other parts of Europe and the USA.

At the meeting Dennis Eriksson from the Swedish University of Agricultural Science reported that the situation in European legislation was in limbo as EU Member States awaited a decision from the European Court of Justice (ECJ) regarding the regulatory status of crops generated by mutagenesis techniques. He suggested that there was cause for optimism following a published opinion from the Advocate General indicating that plants generated by GE should be regulated similarly to those generated by other forms of mutagenesis (Abbott 2018). However, since the meeting, the ECJ has now ruled that crops generated by modern mutagenesis techniques (such as GE) should be regulated under the 2001 GMO directive (Curia.europa.eu 2018). This decision has been criticised by plant scientists as it states that assessments of crop safety should continue to be made on the basis of process rather than product, regardless of the safety of the final variety (Leyser 2018, EPSO 2018).

Louise Ball from the UK Department of the Environmental and Rural Affairs (DEFRA) spoke positively about use of GE, and the UK Advisory Committee on Releases to the Environment (ACRE) has approved the application for a field trial of a gene edited *Camelina sativa* at Rothamsted Research since the meeting (Rothamsted Research 2018). In the opening line of

its decision, ACRE '*considers that Camelina sativa plants produced by CRISPR-Cas9 genome-editing could have been produced through traditional breeding techniques*' (GOV UK 2018). This outcome indicates that UK regulators are well disposed toward the use of GE in the generation of novel crops. Whilst Louise Ball stated that DEFRA will take its lead from the ECJ decision, there may be opportunities for future use of this technology in the UK post-brexite. The ECJ decision does include provision for EU countries to make their own decisions on use of crops generated by any mutagenesis technique. Therefore, it is likely that the ruling will not be applied evenly across the continent.

Stefan Jansson (Umea University) provided the opening keynote to the Bristol meeting, outlining how the Swedish Board of Agriculture does not consider GE plants any differently from those generated by conventional mutagenesis techniques (Eriksson D, 2018). Stefan Jansson has travelled through different Nordic countries to showcase the first gene-edited meal, and highlighted challenges that will arise if countries differently interpret the GMO directive, now encompassing GE plants. Whereas in Sweden the entire GE plant can be grown, in Finland only certain portions of the plant are permitted; whilst Norway, which is not an EU country but which adheres to the EU GMO directive, only allows plant GM products but not the actual plant. The Norwegian Biotechnology Advisory Board has recently proposed a set of regulations that would place all mutagenic events (including those used in conventional breeding or GE) and cisgenic events at a regulatory level below that used to regulate transgenic plants. Stefan pointed out that regulatory bodies will not be able to discriminate between CRISPR-edited and mutagenised plants on the basis of end-point sequence data.

Light-touch regulation in the USA

Professor Gary Marchant from Arizona State University reported at the meeting that in contrast to Europe's process-based and heavy handed regulatory environment, the US regulatory environment for use of gene-edited crops is light-touch. US Department of Agriculture (USDA) regulation of GM crops centres on whether the plant contains any 'plant pest DNA', commonly including viral promotor sequences. If so, approval of the crop requires a laborious and prohibitively expensive process, stifling innovation in Small and Medium-sized enterprises (SMEs). However, the regulation of GE has taken a surprising twist. As transgene-free GE organisms do not contain plant pest DNA, they cannot be regulated by the USDA, and equally do not fall under the regulatory auspices of any other US regulatory agency such as the Food and Drug Administration (FDA) and the Environmental Protection Agency, (EPA).

A growing number of gene edited plants has now been approved by the USDA, following verification that they contain no transgenic DNA (USDA 2018). This rapid approval process might appear attractive to scientists who want to quickly bring products to market, but the consensus from meeting delegates was that risk and evidence-based regulations are preferable to very light touch or no regulation (Emily Waltz 2018). If the US regulatory position is taken to its logical conclusion, then a GE plant that generates a known toxin could be approved as it does not contain 'plant pest DNA'. Therefore, a product that has had little testing may generate unforeseen harmful by-products, which could damage future use of this technology and

compromise public confidence. Gary conceded that in the USA, this issue is likely to remain unresolved in the near term.

As trading nations take different stances regarding the growth and use of GE crops, and it may be impossible to discriminate between GE crops and crops that have been engineered by mutagenesis or bred by conventional methods, it is likely that cross-border regulation of these products will be unworkable and unenforceable.

Technical considerations relating to gene editing

Removal of transformation bottlenecks

Irrespective of the above regulatory uncertainty, GE technology provides an unsurpassed opportunity to modify gene function. Transformation of many crops occurs at low efficiency, and many other plants are recalcitrant to genetic transformation. GE has potential to obviate the requirement for genomic transgene integration in the production of stable mutant lines. Whilst DNA delivery into plant cells is a bottleneck in GE, a few speakers reported progress in removing this bottleneck. Heather Whitney at the University of Bristol gave an enlightening talk on carbon nanodots, which are easy to make, non-toxic and can be functionalised to deliver DNA into plant cells. Her lab and colleagues at Bristol are currently trialling use of this technology to express Cas9 and sgRNAs in a range of grass crops including wheat, and there is exciting potential for broad application across the plant tree of life.

Choun-Sea Lin travelled from Taiwan to discuss a protoplast transformation technique that has shifted the bottleneck in generating mutants from DNA delivery into cells and transformation to efficient regeneration in tissue culture. He discussed polyethylene glycol delivery of DNA into protoplasts, regeneration and editing of 11 species (5 grasses, 4 brassicas, and 2 nightshades) and has developed methods to screen individual protoplasts for edits (Figure 1, Lin *et al.*, 2018). Choun-Sea's group is eager to use their expertise on an expanded number of plant species and welcomes collaboration.

Best practice in construct design

Although many labs are using CRISPR, best practice is not yet fully established. Baptiste Castel from Jonathan Jones' lab at The Sainsbury lab, Norwich presented a comprehensive assessment of the molecular parameters for using the CRISPR system in Arabidopsis. Their lab has determined that either UBI10, YAO or RPS5 promoter-induced expression of a plant-optimised Cas9_3, which includes an internal intron, caused the highest mutation rates. In addition they produced a more efficient 'extension-flip' variety of the guideRNA. Finally when the Cas9 and guideRNA were positioned head-to-head within a T-DNA, they could isolate more stably edited plants in the T1 generation (Figure 2).

Michaela McGinn (Illinois State University) reported that, in the novel oilseed feedstock crop Pennycress, the source of the Cas9 nuclease partially determined editing efficiency. She found

that *Staphylococcus aureus* Cas9 had higher editing rates than *Staphylococcus pyogenes* Cas9, and that Cas9 expression levels as determined by western blot were not predictive of editing efficiency for either nuclease.

Screening

Michaela McGinn also reported that edits did not always appear in or stabilise in the T1 generation, sometimes appearing in T2 or later generations. Thus, transformed plants that initially showed WT gene activity may in fact generate *de novo* edits that result in mutant phenotypes in the T2 generation (Figure 3). Such edits were detectable in sequence traces from T1 GE plants, and trace analysis was therefore recommended. Michaela had used CRISPR-Cas9 to reduce the levels of an undesired fatty acid from 40% in wild-type Pennycress seed to less than 1% in GE mutants (McGinn *et al* 2018)÷ .

A potential concern with the use of CRISPR-Cas9 to create new crop varieties involves the generation of off-target edits across the genome. To allay these concerns, Yiping Qi from the University of Maryland described a remarkable set of experiments that involved conducting whole genome sequencing on almost 70 individual rice plants (Tang *et al.*, 2018). This allowed them to assess the amount of spontaneous mutations in unedited plants in comparison with those that had been edited by either Cas9 or Cpf1 nucleases. Yiping Qi showed that any allele changes could be explained by the rate of spontaneous mutations and that neither of the nucleases induced additional off-target mutations in the T1 generation.

Given the technical challenges of GE, it might be more cost-effective for researchers to engage with a community resource, and a number were introduced throughout the meeting. These include facilities at the National Institute for Agricultural Biology, Cambridge, the University of Bristol, Rothamsted Research, the John Innes Centre and the Vienna Biocenter Core Facility. Each facility welcomes prospective collaborations.

Novel applications of gene editing nucleases

Whilst the meeting focussed on GE, novel uses for Cas9 gene targeting were also discussed. Alex Leydon from Jennifer Nemhauser's group at the University of Washington in using repurposed nucleases for transcriptional control. The synthetic HACR protein includes a dCas9 that binds DNA but lacks nuclease activity, a transcriptional repression domain and a hormone-inducible degron (Khakhar *et al.*, 2018). By either replacing the repression domain and/or the degron, the HACR system can provide enormous variation in experimental design. The core dCas9 subunit allows tight gene regulation, and the Nemhauser group has used different degrons to generate a set of hormone biosensors that are responsive to auxin, jasmonate or gibberellic acid (GA). Although at the workshop Alex specifically described research that had modeled the response to GA, enormous potential of the HACR system was clear.

Conclusion

This meeting introduced a wide range of projects that are using CRISPR-based GE and highlighted attempts to optimise GE technology. The community is progressing towards the establishment of a set of standardised protocols that will reduce challenges for new users. Rarely has the future use of a technology been so tightly linked to its regulation and this meeting highlighted the policies employed by different countries. Although a global consensus for evidence- and trait-based regulation of gene edited crops seems some way off, differences in how countries apply these rules will hopefully mean that the technology can still be used to develop useful and much needed novel varieties.

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Figure Legends

Figure 1: Assessing gene editing in single isolated protoplasts.

Protoplasts were released from a single leaf and diluted to a concentration of 1 cell/ul. Following DNA delivery, screening involved two rounds of PCR and dCAPs analysis to detect gene edited alleles, or plants were regenerated. Adapted from Choun-Sea Lin from the Academia Sinica, Taipei.

Figure 2: Optimised CRISPR-Cas9 system for gene editing in Arabidopsis.

A: Gene editing construct design favoured by Baptiste Castel from The Sainsbury lab, Norwich.

Figure 3: Scoring a sequencing trace from T1 gene edited plants.

Traces from top down:

A: a true wildtype sequence with the *Staphylococcus pyogenes* Cas9 NGG PAM site boxed in red. B" a 'wildtype' sequence with low level editing activity indicative of stable indels in subsequent generations. C: a heterozygous Cas9 generated edit. D: a homozygous edit (+A insertion event). E: a bi-allelic edit from two independent editing events in a single generation.

Figure 1

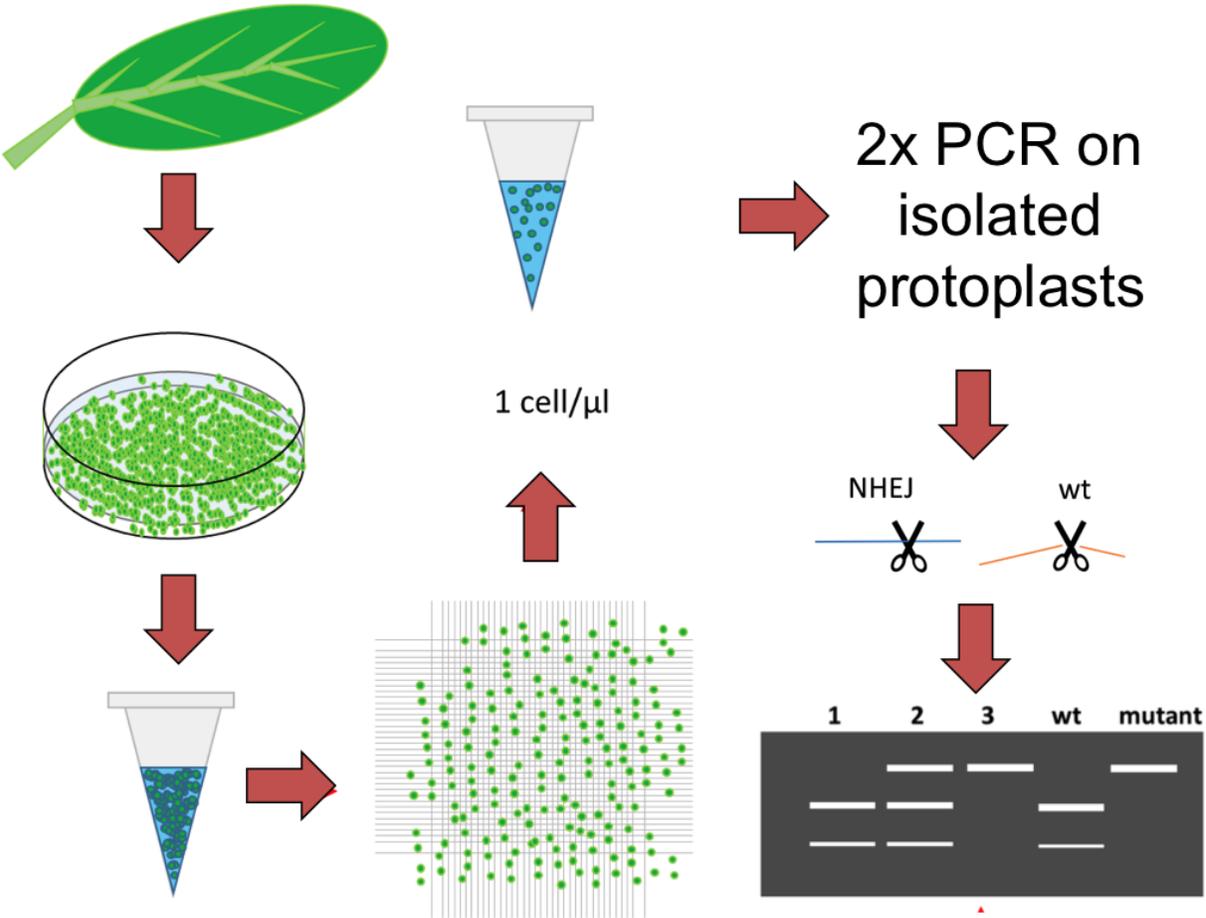


Figure 2

Best orientation = Head-to-head
Best promoters = RPS5a, YAO and UBI10
Best Cas9 = Cas9_3 (with an intron)
Best Cas9 terminator = Ocs and E9
Best sgRNA = Extension-Flip
Best sgRNA terminator = Long Terminator

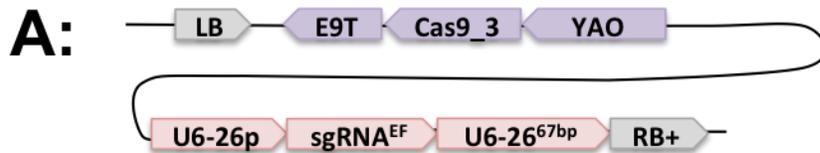


Figure 3

