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PERSPECTIVE

Metabolomics should be deployed in the identification and characterization of gene-edited crops

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SUMMARY

Gene-editing techniques are currently revolutionizing biology, allowing far greater precision than previous mutagenic and transgenic approaches. They are becoming applicable to a wide range of plant species and biological processes. Gene editing can rapidly improve a range of crop traits, including disease resistance, abiotic stress tolerance, yield, nutritional quality and additional consumer traits. Unlike transgenic approaches, however, it is not facile to forensically detect gene-editing events at the molecular level, as no foreign DNA exists in the elite line. These limitations in molecular detection approaches are likely to focus more attention on the products generated from the technology than on the process in itself. Rapid advances in sequencing and genome assembly increasingly facilitate genome sequencing as a means of characterizing new varieties generated by gene-editing techniques. Nevertheless, subtle edits such as single base changes or small deletions may be difficult to distinguish from normal variation within a genotype. Given these emerging scenarios, downstream 'omics' technologies reflective of edited affects, such as metabolomics, need to be used in a more prominent manner to fully assess compositional changes in novel foodstuffs. To achieve this goal, metabolomics or 'non-targeted metabolite analysis' needs to make significant advances to deliver greater representation across the metabolome. With the emergence of new edited crop varieties, we advocate: (i) concerted efforts in the advancement of 'omics' technologies, such as metabolomics, and (ii) an effort to redress the use of the technology in the regulatory assessment for metabolically engineered biotech crops.

Keywords: genome-editing, metabolomics, crop regulation, food system, substantial equivalence.

INTRODUCTION

The last decade has been characterized by the adoption of genome-editing systems following the revolutionary discovery of transcriptional activator-like effector (TALE) proteins, which are more suitable for the precise engineering of targeted DNA sequences (Stella and Montoya, 2016) and the subsequent widespread adoption of the clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein (Cas) system (Puchta and Fauser, 2014; Scheben *et al.*, 2017; Yin *et al.*, 2017). We refer the reader to the plethora of excellent reviews on plant gene editing (Kim and Kim, 2014; Baltes and Voytas, 2015; Bortesi *et al.*, 2016; Wright *et al.*, 2016; Pacher and Puchta, 2017; Zhu *et al.*, 2017; Knott and Doudna, 2018; Langner *et al.*, 2018) for details of the underlying molecular mechanisms and the manifold applications. Here we outline the opportunities that these techniques afford as well as review recent additions to the repertoire of applications. We then discuss prevailing legal and political viewpoints regarding the introduction of gene-edited crops into agricultural production, before proposing a bi-functional role of metabolomics technologies in the regulatory assessment of gene-edited crops. For context, we first provide a brief review of current metabolomics techniques and their prior use in the evaluation of transgenic plants. We argue that metabolomics represents an efficient means to discriminate gene-edited and non-edited control plants, and is an important tool for assessing both intended and unanticipated metabolic outcomes, which could suggest the need for further characterization. Finally, we provide a perspective that we anticipate will aid in establishing a road map for metabolomics-based identification and safety assessment of gene-edited plants, taking into account potential environmental effects and interactions with the metabolome. Whether achieved via gene editing, induced or natural mutation, or from available genetic diversity, the critical question remains: if a biochemical step or component of a physiological process is altered, what is the final outcome in chemical composition of the product and how will the scientific community detect, quantify and communicate these changes?

THE ADVENT AND ADOPTION OF GENOME EDITING IN PLANTS

Genome editing was initially achieved using meganucleases with target sites of up to 18 bp in length; however, the fact that the double-stranded breaks produced by these enzymes were determined by the natural specificities of the enzymes made them difficult to work with (Rosen *et al.*, 2006). As a consequence, designer nucleases with specificity characteristics, such as zinc-finger nucleases and transcription activator-like effector nucleases were developed and overcame this problem (Kim and Kim, 2014; Zhu *et al.*, 2017). The most recent technology is based on a form of bacterial adaptive immunity, in which previously encountered invasive DNA sequences are committed to molecular memory and targeted in future challenges by expressing CRISPRs representing DNA fragments captured from invading pathogens. The resultant CRISPR RNAs act as guides for CRISPR-associated (Cas) nucleases that attack the pathogens upon subsequent infection (Knott and Doudna, 2018; Langner *et al.*, 2018). Researchers have exploited this process by constructing synthetic guide RNAs that direct the Cas nuclease to genomic targets. The benefits and applications of genome editing include its greater precision without the presence of foreign DNA in the edited genome. Although the technique has been much reviewed in recent years (Baltes and Voytas, 2015; Puchta, 2017; Scheben *et al.*, 2017; Komor *et al.*, 2017a; Kumlehn *et al.*, 2018; Scheben and Edwards, 2018), it is imperative to realise that our understanding of both its scope and the mechanisms by which it can operate are rapidly evolving. For example, targeting precision has recently been improved in plants and other organisms by a range of means, including the use of different CRISPR-associated nucleases (Komor *et al.*, 2017b; Kim *et al.*, 2019; Raitskin *et al.*, 2019), in addition to now achieving editing without the need for double-stranded DNA breaks (Gaudelli *et al.*, 2017). Enhanced precision should reduce so-called 'off-target' effects, whereby genes other than that targeted gene are edited. The lack of a need for double-stranded DNA breaks bypasses the need for homologous recombination, greatly increasing the efficiency of editing (Gaudelli *et al.*, 2017). Other technological developments of note include a microRNA-inducible CRISPR-Cas9 platform that can act as a microRNA sensor and cell type-specific tool for genome regulation (Wang *et al.*, 2019), one-step genome editing during haploid induction (Kelliher *et al.*, 2019), and the development of strategies for gene replacements and insertions by intron targeting (Li *et al.*, 2016). Moreover, gene editing has been demonstrated to have highly versatile applications, including its use in generating fertile transplastomic Arabidopsis plants (Ruf *et al.*, 2019), in the novel domestication of crops (Lemmon *et al.*, 2018; Li *et al.*, 2018; Zsogon *et al.*, 2018) and in the re-evaluation of a classical ripening mutation in *Solanum lycopersicum* (tomato; Ito *et al.*, 2017). Its potential for agriculture is both astounding and expanding. In summary, already at this early stage (Fernie and Yan, 2019), gene editing can be used to: activate or suspend the function of a gene (Qi *et al.*, 2013); create multiple different alleles of a gene (Rodriguez-Leal *et al.*, 2017); edit any base (Gaudelli *et al.*, 2017); repair deletions (Dahan-Meir *et al.*, 2018); add genes that do not exist in the original genome (Park *et al.*, 2014); and delete any sequence, including large chromosomal fragments or even entire chromosomes (Xiao *et al.*, 2013). CRISPR has successfully been used in many important crop species including, but not limited to, the cereals

Hordeum vulgare (barley; Lawrenson *et al.*, 2015), *Oryza sativa* (rice; Shan *et al.*, 2013; Li, *et al.*, 2016), *Triticum aestivum* (wheat; Shan *et al.,* 2013; Zhang *et al.*, 2016) and *Zea mays* (maize; Shi *et al.*, 2017), as well as *Brassica oleracea* (Lawrenson *et al.*, 2015), *Citrus* (Peng *et al.*, 2017), *Cucumis sativus* (cucumber; Chandrasekaran *et al.*, 2016), *Glycine max* (soybean; Demorest *et al.*, 2016), *Solanum lycopersicum* (tomato; Cermak *et al.*, 2015), and *Solanum tuberosum* (potato; Clasen *et al.*, 2016; Ye *et al.*, 2018), with applications in novel species published on a monthly, if not weekly, basis. In terms of agricultural application, it is essential to realise that that the Cas9 enzyme is, in effect, a biological mutagen, a fact that needs to be conveyed more clearly to politicians as well as to the general public.

In 2018 the European Court of Justice (ECJ) ruled that gene-edited crops should be subjected to the same stringent regulations as those presently in place for genetically modified (GM) organisms (Bobek, 2018). Although a setback for practitioners in the field, who felt that the absence of foreign DNA in the crop would alleviate the need for lengthy regulation processes, the ruling has accentuated the need for the detailed characterization of novel foods and has potentially shifted the focus from detection to a greater need for product characterization, more akin to current US regulation. For example, most countries who have not taken the same stance as Europe and want to empower the potential of the technology will be developing and releasing a plethora of new alleles into the supply chain. Considering the potential similarity to existing alleles and the limitations of genome sequencing in assessing gene-editing events, how will these changes be detected? Advanced metabolomics could have a role in rapidly determining global changes to the metabolome and how these changes relate to the chemical composition of existing benchmarked varieties/foodstuffs. In addition, classical mutagenesis remains the only technological exception to the regulatory procedures surrounding GM technologies. This criterion is based on a safe history of use. Therefore, it is important to actively engage in discovery and feasibility studies using gene-editing techniques to ensure a wealth of robust scientific data is generated to support these emerging technologies.

METABOLOMICS

The first papers on metabolomics in plants – the comprehensive description of the small molecule complement of the cell – were published over 20 years ago (Katona *et al.*, 1999; Fiehn *et al.*, 2000; Roberts, 2000; Roessner *et al.*, 2001; Aharoni *et al.*, 2002; Le Gall *et al.*, 2003; Weckwerth, 2003; Deferenez *et al.*, 2004; Saito and Matsuda, 2010); however, current methodologies only cover a small percentage of the 200 000–1 000 000 metabolites anticipated in the plant kingdom (Dixon and Strack, 2003; Rai *et al.*, 2017). Three methodologies are commonly used for plant metabolomics: nuclear magnetic resonance (NMR), gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS) (Obata and Fernie, 2012), with LC-MS providing the most comprehensive outcomes (Alseekh and Fernie, 2018). Twin improvements afforded by ultra-performance LC (UPLC) and high-resolution mass spectrometry rendered this technique even more powerful with regards to resolution, sensitivity and throughput (Fernie and Tohge, 2017). Indeed, data on over 1000 metabolites are accessible using either direct infusion or coupled UPLC high-resolution MS (Aharoni *et al.*, 2002; Kind and Fiehn, 2007; Giavalisco *et al.*, 2011). High-resolution MS provided a massive boon to metabolomics in providing sufficient mass accuracy to allow the determination of the exact chemical composition, but not the unambiguous structure assignment of each analyte. Multiple rounds of MS can aid in structural assignment via the identification and assembly of the resultant metabolite fragments, whereas metabolite purification can allow metabolite absolute structure identification via NMR (see for example Tohge *et al.*, 2016). The generation of large libraries of spectra based on authentic chemical standards (Shahaf *et al.*, 2016), isotope labeling (Giavalisco *et al.*, 2011; Nakabayashi and Saito, 2017; Feldberg *et al.*, 2018), MS imaging (Sturtevant *et al.*, 2016; Dong *et al.*, 2016) and the development of a large range of bioinformatics tools (Perez de Souza *et al.*, 2017), including molecular networking approaches (Kang *et al.*, 2019; Perez de Souza *et al.*, 2019), is additionally aiding in filling gaps in the coverage of metabolomics techniques. Enhanced capacity for metabolite identification is important to help biologically interpret any changes observed, but in the context of metabolomics screening for the occurrence of editing events, annotation is actually not necessary. This is in contrast to environmental safety issues, as mentioned by Hall and de Maagd (2014), where our current limited ability for metabolite annotation still restricts our assessment of the overall biological relevance of any observed changes, whether predicted or not.

Metabolic profiling has seen great utility within systems-biology approaches aimed at providing a more comprehensive understanding of plant responses to environmental and genetic perturbations (Roessner *et al.*, 2001; Fernie *et al.*, 2004; Saito and Matsuda, 2010; Weckwerth, 2003). Additionally, metabolic profiling has been used in multiple studies aimed at the safety assessment of GM crops (Kuiper *et al.*, 2001; Christ *et al.*, 2018): e.g. to demonstrate the substantial similarity between conventional potatoes and potatoes engineered to produce fructans (Catchpole *et al.*, 2005), with similar results obtained from metabolomics on field-grown transgenic barley in comparison with wild-type barley (Kogel *et al.*, 2010). Other studies have demonstrated considerable metabolic consequences of genetic alterations in potato (Shepherd *et al.*, 2015) and wheat (Baker *et al.*, 2006), however. Moreover, a recent study

using metabolomics revealed that even the commonly used bialaphos resistance gene (*BAR*) for transgene selection has effects on amino acid levels in a range of species as a result of enzyme promiscuity (Christ *et al.*, 2017). A further study looking at substantial equivalence in tomatoes revealed that the majority of the transgenics exhibited only small changes in metabolomics yet displayed reproducible transformation-related metabolic signatures. This study is thus illustrative of the challenge also present for metabolomics analysis of gene-edited or indeed even conventionally mutated plants (Garcia *et al.*, 2016), in that metabolic changes are likely to be present following genetic perturbation. In the case of gene-edited plants, we believe that this is an opportunity rather than a problem, in that the metabolic changes represent a possible route for the identification of gene-edited individuals. This will clearly depend on the gene-editing event having detectable metabolic consequences. Although an estimated 25–30% of plant genes directly encode metabolically-associated proteins, considerable cumulative evidence from a broad number of studies suggests that the modified expression of most genes will have metabolic consequences (Fernie *et al.*, 2004; Lu *et al.*, 2008). The simplest route for identifying genetic perturbations in the case of metabolism-associated genes would be the identification of alterations in the levels of the products and substrates of the reaction that the proteins they encode catalyze. Following cross-over theory, the deficiency of an enzyme would result in the accumulation of its substrate(s) and depletion of its product(s), whereas the upregulation of an enzyme activity would result in the opposite changes (Newsholme and Start, 1973). This theory only holds true for a simple linear metabolic pathway, which is relatively rare in the case of plant metabolism, however. As mentioned for the examples above, pleiotropic changes in metabolism and changes propagated throughout the metabolic network appear to be relatively common. As such, the combination of multivariate statistics and high-resolution metabolomics are likely to prove instrumental as a means of discriminating gene-edited plants from their wild-type controls as well as from spontaneous mutants or early generations of physically or chemically induced mutants, which are likely to contain multiple mutations. Considering the sensitivity and comprehensiveness of the technology for detecting metabolic differences, it is important to emphasize that all metabolic comparisons must be made under correct and fully controlled environmental conditions, in order to permit the proper robust comparison of samples where only the genetic component can be causal to any differences observed.

PERSPECTIVE

In recent years funding bodies have promoted multidisciplinary and interdisciplinary research that involves input from the disciplines of engineering, natural sciences, social sciences and humanities, in such a manner that no single discipline predominates. Co-design has now become a term and practice that appears concurrently on some of the most cutting-edge scientific proposals, in an attempt to deliver better societal perception, transparency and acceptance of new technologies (Steen *et al.*, 2011). It is also important and necessary to have robust scientific data taken over a prolonged period of study to ensure that a truly informed judgement can be made, however. For example, despite the lack of consumer acceptance, it is impressive that the vast body of multidisciplinary outputs have been able to establish first-generation transgenic crops as a safe technology. Given this endorsement of scientific robustness and approaches, is it not sensible to treat the emerging technologies of gene editing in crop plants with the same rigor as previously carried out with transgenic technologies? In fact, we can learn from previous successful approaches. The precision of gene editing also poses new challenges to detect new edited alleles in germplasm across the supply chain. With this in mind, metabolomics or even 'next-generation metabolomics' (e.g. with greater annotation of metabolites with high confidence) has a key role to play in the new paradigm of gene-edited crops, especially if there is a shift to product characterization rather than the production process.

Perhaps one of the first questions that must be asked is: can we rapidly detect a change in the metabolome? An untargeted metabolite analysis component of a hierarchical workflow clearly has utility in this respect. Subsequently, there is a requirement to identify the indirect biochemical changes globally and the precise reaction/process affected. In comparison to genomics, it is clear that metabolomics has not proceeded with the same rapidity. In part because of the intrinsic diverse and dynamic chemical nature of the metabolome. Despite these limitations, the importance of the metabolome in representing the outputs of cellular processes and the chemical composition of foodstuffs, there is a genuine necessity to improve our ability to determine metabolome composition. How could this be achieved in a generic biological context? Clearly metabolite identification via species-specific quantitative data and chromatographic annotation needs addressing. In addition, pre-purification on the basis of polarity and the development of spatial metabolomics would help to overcome the issue of dynamic range and incorporate more biological texture to the data. Likewise, advanced tools for the better integration of multi-omic data sets would be beneficial. The fact that genome-wide association study populations are rapidly identifying the genetic architecture of known metabolites suggests that this approach will gain utility in identifying unknown metabolites in the future. In conclusion, the emerging technologies associated with gene editing in crops will be revolutionary to both fundamental and applied science. The advances only highlight the need for

the concurrent development of approaches to analyse, characterize and detect changes across biochemical systems in an unbiased manner, however.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

There are no data associated with this article.

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