

to only one cell type. In this case, the monoclonal antibody is produced only in the tubular-gland cells of the reproductive tract, which are the site of ovalbumin synthesis. The importance of restricting production of ectopic protein to one cell type is twofold. From a physiological perspective, there is a significant risk of negatively impacting the health and welfare of the birds when a protein that is normally restricted to a limited number of physiological compartments is produced ubiquitously. From a product quality perspective, restricting production to a single tissue reduces the heterogeneity of the glycoforms of the protein. For example, the monoclonal antibody that we produced, like the majority of egg white proteins secreted by the tubular-gland cells, does not possess fucose. By contrast, antibodies produced by B cells in the chicken are fucosylated [3], and a proportion of these antibodies would be passively transferred to egg white if ubiquitous promoters were employed.

The absence of fucose alters the therapeutic utility of the monoclonal antibody by significantly increasing antibody-dependent cellular cytotoxicity (ADCC) activity. ADCC is a part of the mode of action of several therapeutic antibodies currently in use (e.g. Herceptin and Rituxan); therefore, the increase in ADCC is an attractive attribute of the chicken tubular-gland cell-produced antibody. The increase in potency associated with the enhanced ADCC reduces the dosage of antibody that is required and, therefore, the number of eggs required to manufacture each dose.

The egg white contains ~3 g of protein, and ~54% of the egg white is ovalbumin. About 800 mg of ovalbumin can, therefore, be attributed to each allele. Although others have suggested deposition of as much as 1 g of transgenic protein per egg, we believe that this is unlikely given the history of animal transgenesis, and ill advised from the point of view of egg production. Adding 1 g of a soluble protein to the highly viscous egg white before the shell membrane is deposited might be incompatible with normal egg formation.

We share the enthusiasm of Dr Ivarie for making therapeutic proteins in the eggs of chickens. As we have demonstrated, the use of large transgenes that confer tissue-specific expression and induce high rates of protein production could make the egg the vehicle of choice in the manufacture of therapeutic monoclonal antibodies.

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Research Focus

Unintended effects in genetically modified crops: revealed by metabolomics?

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In Europe the commercialization of food derived from genetically modified plants has been slow because of the complex regulatory process and the concerns of consumers. Risk assessment is focused on potential adverse effects on humans and the environment, which could result from unintended effects of genetic modifications: unintended effects are connected to changes in metabolite levels in the plants. One of the major challenges is how to analyze the overall metabolite composition of GM plants in comparison to conventional cultivars, and one possible solution is offered by metabolomics. The ultimate aim of metabolomics is the identification and quantification of all small molecules in an organism; however, a single method enabling complete metabolome analysis does not exist. Given a

comprehensive extraction method, a hierarchical strategy – starting with global fingerprinting and followed by complementary profiling attempts – is the most logical and economic approach to detect unintended effects in GM crops.

Introduction

Food derived from genetically modified (GM) crops is often perceived disparagingly by consumers – partly because of concerns about unintended effects (Box 1) on human health [1]. As a result, guiding principles and regulatory frameworks have been established for safety assessment [2], the main tenet of which is ‘substantial equivalence’ or better ‘comparative safety’. According to this concept, the safety of a GM crop can only be established by comparison

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Box 1. Genetically modified plants – from intended to unintended effects (modified from [16])

Intended effects

- effects that are targeted to occur after gene transfer has been successfully accomplished
- lead to improvements in phenotype, composition or agronomical traits
- metabolites of interest can be analyzed by targeted quantitative methods

Unintended effects

- effects which represent a statistically significant difference (e.g. in chemical composition of the GM plant compared with a suitable non-GM plant grown under the same conditions)
- the evaluation should take the biological variation of the comparator into account and, therefore, an appropriate number of background samples has to be analyzed
- changes might have an impact on potential agronomic performance but they do not necessarily pose safety threats for human health or the environment

Predictable

- effects which are unintended but could be expected based on our current knowledge on plant metabolite pathways, gene-to-gene interactions and general plant biology
- the class of influenced metabolites is predictable, thus targeted quantitative methods can be applied
- allergenicity is often discussed in this context

Unpredictable

- effects that represent a statistically significant difference (e.g. in the chemical composition of the GM plant compared with the non-GM plant), and could not be expected in advance based on our current understanding
- metabolic profiling methods are suitable to provide information, even concerning small but potentially important changes

with a comparator with a history of safe use (e.g. the traditionally bred parent in the case of vegetatively propagated crops) in terms of morphology, agronomic characteristics and composition comparisons. The differences in the composition form the basis for a series of toxicological and nutritional studies designed to evaluate the impact of consumption by humans.

Natural genetic variation within species, and between related species, has been a major source for crop improvement. For centuries conventional plant breeding programs have produced new traits, higher yields and improved quality. However, little attention has been paid to metabolic changes occurring in successive generations. This issue has gained importance only recently in the context of defining thresholds for safety assessments of GM crops.

Non-targeted, analytical approaches at the gene, transcript, protein and metabolite levels are the methods-of-choice for investigating the physiology of the GM plants as comprehensively as possible, thus increasing the chances of detecting unintended effects. In a recent paper [3], the application of metabolomics methodology has been shown to be useful for the investigation of compositional similarity in GM potatoes. These results are particularly exciting because they also give profound insights into the extent of variation within conventional cultivars.

How do unintended effects arise?

Despite significant advances in direct or indirect gene transfer techniques, genetic engineering is still somewhat dependent on erratic rather than predictive effects. This is because transgene integration in plants occurs through illegitimate recombination. DNA integration is random, with a preference for gene-rich regions, and gene disruptions, sequence changes and the production of new proteins can occur as a consequence of the recombination event. If one accepts single-gene transfer and hybridization as two extremes of the same approach, it is not surprising that there are also unintended effects in conventional breeding: in breeding programs, a significant proportion of time is usually spent backcrossing in an attempt to eradicate introduced undesirable traits.

Metabolic networks display a high degree of connectivity, as has been shown for *Saccharomyces cerevisiae* where ~50% of the metabolites are involved in more than two reactions, and more than 67% of all reactions involve more than one substrate and product [4]. Despite, or because of, this connectivity, it cannot be deduced that a higher number of introduced genes results in more unintended effects. Rather, the opposite has been shown in a case where the genes encoding a whole metabolon (for dhurrin biosynthesis) have been transferred to naturally acyanogenic *Arabidopsis* [5]. Alternatively, single-gene products might interact more efficiently with other pathways – a principle that is exploited in combinatorial biochemistry approaches [6].

Preconditions for comprehensive chemical analyses

Completely unbiased chemical analysis is virtually impossible because it would require non-invasive techniques throughout. The analysis of plant metabolites is generally complicated by their highly complex nature and enormous chemical diversity. Nevertheless, is it now possible to choose from a variety of techniques, such as flow injection electrospray ionization mass spectrometry (FIE-MS), Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), Fourier transform infrared (FTIR) spectroscopy and nuclear magnetic resonance (NMR), which facilitate rapid fingerprinting of crude extracts, thus limiting sample treatment to a minimum. However, even a non-selective method can only characterize the compounds in the sample that have been reliably extracted from the plant matrix. It is, therefore, important to bear in mind that all extraction techniques constitute a compromise. The parameters for certain compound classes can be optimized, specifically, if a targeted approach is applied, whereas broad coverage is the highest priority for non-targeted analysis – at the price of potentially low extraction efficiency for some metabolites, which might render them undetectable in the analysis. At the metabolome level, the problem can be circumvented by fractionated extraction and subsequent analysis of the entire polarity range (from non-polar to polar compounds) [7]. Major techniques for metabolomics, such as the identification and quantification of as many compounds as possible, are gas chromatography (GC) and liquid chromatography (LC), coupled to MS detectors. These techniques are complementary because of their

intrinsic limitations. LC-MS permits the direct analysis of compounds with a wide range of polarities but results in less stable retention times, which complicate the automatic data processing, whereas retention times are extremely reproducible in GC-MS. Even compounds with identical retention times can be quantified by selecting characteristic ions. The main disadvantage of GC is the need for derivatization to analyze polar (non-volatile) metabolites. This means that compounds that are not successfully derivatized, and those with molecular weights outside the scanned mass range, remain undetected. Complementary sampling techniques, such as solid-phase microextraction (SPME), permit the integration of non-derivatized volatile compounds [8], which are often neglected, despite comprising important flavor and fragrance determinants.

Handling the data

Instrumental analysis constitutes only one task of metabolomics projects – of at least equal importance is the statistical data treatment. Data processing algorithms need to be applied to detect peaks in spectral data, match the corresponding peaks across the samples and correct the peak intensities for instrumental variability (normalization). This permits the comparison of relative levels of compounds in samples even if the metabolite identity is unknown [9]. As previously outlined in the context of safety assessment procedures, the composition of GM crops has to be evaluated against a background of natural variability in conventional cultivars: a statistical problem similar to diagnostic investigations of metabolic disorders in humans [10]. Usually the extent of compositional variation in traditional crops is unknown. However, until databases and legally binding thresholds are available, the discussion on ‘safe’ limits continues. Among the multivariate statistical models are supervised and unsupervised techniques, permitting the visualization of clusters or classes of metabolites in the search for unintended effects [11]. As for the instrumental analyses, their applicability has to be evaluated on a case-by-case basis.

It is all about integration

Plants are complex organisms. We are just beginning to understand this following the sequencing of the first model plant in 2000 [12]. *Arabidopsis* has more genes than previously thought and the number in many crop plants with more complex genomes will be even higher [13]. Although the metabolome cannot be computed from the genome [14], it is probable that the estimated number of metabolites produced by plants is rather conservative because only a small percentage of all currently known plant species has been chemically investigated with respect to secondary metabolites [15].

Many closely related molecules can be obtained from the same substrate depending on the metabolic state of the cell. Moreover, similar compounds often possess different biological activities. Therefore, potentially harmful metabolites that accumulate in GM plants as a result of an unintended unpredictable effect need to be carefully evaluated. One of the key challenges of metabolite analysis is to find an optimal balance between accuracy

and coverage of metabolite measurements. Catchpole and co-workers [3] demonstrated the compositional similarity between GM and non-GM potatoes using mass spectrometric fingerprinting as a primary screen, which was then amended by detailed quantitative profiling analyses. This approach (i.e. combining non-targeted and targeted analytical methods) appears feasible even for screening large numbers of transgenic plants, and the application of both supervised and unsupervised data analysis techniques ensures independency from statistical bias.

Monitoring changes at the gene, transcript, protein and metabolite level in GM crops, as required by the safety assessment procedures, will result in enormous amounts of data. The main challenge in the future will be to integrate these data in the context of systems biology. Ultimately, this will lead to predictive genetic engineering, which will help to avoid unintended unpredicted effects in GM crops.

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