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Alterations in genetically modified crops assessed by omics studies: Systematic review and meta-analysis

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ABSTRACT

Background: International agreements and domestic legislation regulate genetically modified (GM) crops for environmental release, recognizing that genetic engineering could result in unintended genotypic and phenotypic effects. In that context, omics technologies, which allow comprehensive characterization of the molecular profile of GM crops at all levels, may be used to assess alterations or effects of genetic engineering.
Objective: To determine whether omics techniques are suitable tools to comprehensively screen for metabolic changes due to genetic modification in plants.
Approaches: A literature search was conducted in four online scientific databases for relevant publications. After removal of duplicates, we retained only studies that included *cry*, *epsps* and *pat/bar* transgenes. We evaluated the full texts of the remaining papers and performed data extraction. We placed the extracted outcomes into an evidence table, which comprised six major categories, including an analysis of altered metabolic pathways based on the KEGG pathway database.
Main findings: Sixty articles were included in this review. We found a high proportion of publicly funded studies (86.7%) compared to just three with industry financial support. We found that 40% of the plant material analyzed was produced in the field, 26.7% in growth chambers, and 18.3% in greenhouse experiments, although this information could not be extracted from all studies. More than one third (38.4%) of the studies did not use a non-GM near-isogenic line as a comparator, and half did not specify the number of plants used per sample in their reports. All the studies (except three that did not perform a comparative analysis) reported statistical differences in GM versus non-GM omic profiles. A heatmap analysis showed that the most frequently affected metabolic pathways were related to metabolism of carbohydrates, energy, lipids, and amino acids, as well as genetic information processing and environmental information processing.
Conclusion: This review shows that omics techniques can profile different levels of genetic information and metabolism and can be useful tools in assessing alterations in genetically modified plants. In recent years, there have been intensive efforts to harmonize omics methods. Consistent guidelines with standardized frameworks are needed to capitalize on the unquestionable potential of implementing untargeted omics analyses in the risk assessment process. Finally, there is a need to build an assessment framework connecting omics results to biologically relevant changes in the GM organism, and this framework to be operable for the risk assessment process.

1. Introduction

In general terms, genetically modified organisms (GMO) are organisms that are altered using modern biotechnology techniques, such as in vitro recombination of nucleic acid (DNA or RNA) molecules (Luis La Paz et al., 2014), resulting in an organism with a novel combination of genetic material. In the agricultural sector, genetically modified (GM) crops, such as soybean, maize, cotton and canola, have been widely

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adopted by exporter countries like the U.S., Brazil, Argentina, Canada and India, with an adoption rate reaching more than 93% of their agricultural area in 2018, according to industry sources (ISAAA, 2018). These GM crops have mainly been transformed by either biolistic or *Agrobacterium tumefaciens* -mediated techniques, resulting in the insertion of transgenes into their genomes. Most often, the transgene confers the ability to produce an insecticidal protein (e.g. Cry proteins from *Bacillus thuringiensis* - Cry maize) or tolerate herbicide sprays which would otherwise kill plants (e.g. Roundup Ready soybean – EPSPS soybean) (ISAAA, 2018).

The use and release of GMOs in the environment aroused concerns regarding potential environmental risks since 1992, when article 8(g) of the United Nations Conventional on Biological Diversity (CBD) was written to establish or maintain means to regulate, manage or control the risks associated with the use and release of living modified organisms, which includes GM crops. The provisions of this article inspired the development of a dedicated protocol on biosafety – The Cartagena Protocol on Biosafety. Since then, several guidance documents have been developed to assess the safety of GMOs before they are considered for market approval (UNEP 2014).

In general, the GMO risk assessment is structured in steps beginning with a "hazard identification" phase (Fig. 1). It is frequently composed of molecular characterization of the GM plant (e.g. information related to the genetic modification and the host recipient, such as the DNA sequence of the transgene or insertion site), a compositional analysis, and a description of the agronomic and phenotypic characteristics of the GM crop plant (EFSA, 2011). In the compositional analysis, selected compounds of the GMO are compared with one or more conventional comparators, as formulated by the Food and Agriculture Organisation (FAO), the Organisation for Economic Co-operation and Development (OECD) and EFSA (EFSA, 2008, 2011; FAO 1996; OECD 1993). It includes key compounds that have been described for several species by the OECD Task Force in consensus documents (OECD 2002). The hazard identification step is crucial for the definition of risk hypotheses at later steps in the risk assessment process. Therefore, failure to address potential hazards at early stages leads to the underestimation of risk and, consequently, reduces the risk assessor's confidence that all risk hypotheses have been tested (Heinemann et al., 2011).

However, despite the institutional efforts to adapt to the many biotechnology advancements, safety criteria based on targeted analyses have been criticized for representing a limited number of compounds and for being biased towards certain toxins, antinutrients, or other secondary products and cannot cover unknown molecules arising from the genetic modification (Gong & Wang, 2013; Agapito-Tenfen et al., 2015; Valdes et al., 2014; van Dijk et al., 2014; Hilbeck et al., 2015; Selb et al., 2017; Corujo et al., 2018; Verhoeckx et al., 2019).

Recent developments in different omics techniques have made it possible to characterize organisms' molecular profile in a comprehensive and high-throughput manner. But the increased number of analytes or compunds is not the only advantage of these techniques as it is now possible to capture several layers of information of the organism. The systematic analysis with the aid of sophisticated algorithms and cross-linked databases leads to an unprecedent opportunity to derive dedicated risk hypothesis at very early stages of the risk assessment. This has led to the use of omics technologies to assess alterations in GM crops (Christ et al., 2018; Agapito-Tenfen et al., 2015). In the context of risk assessment, numerous independent studies have used omics techniques (mainly proteomics, metabolomics and transcriptomics) to analyze various GM crops grown under different conditions. The results were inconsistent across the GM events due to the heterogeneity of experimental designs, and, therefore, neither potential alterations nor the underlying mechanisms have been identified, let alone understood (Bridges et al., 2017).

In this article, the use of omics techniques to detect alterations in GM crops is systematically reviewed based on the available studies from 2006 to 2020. Study selection is conducted based on PRISMA guidelines (Liberati et al., 2009). We have focused our analysis on high throughput techniques for transcriptomics, proteomics and metabolomics for the purpose of searching for effects in the plant's metabolism. However, this should not be perceived as our definition of the only omics techniques that may be relevant for risk assessment. For each included omics platform, data was organized by plant material and introduced traits, and then the statistically significant altered metabolic pathways in the research articles were analyzed and illustrated. Empirical evidence that supports the results from omics studies of GMO unintended effects is discussed, and some future research needs and recommendations for omics implementation are proposed. The research question framing this review is: are omics-based molecular profiling techniques suitable tools to comprehensively screen for metabolic changes brought about due to genetic modification? We conducted a literature review to find, collect, and compare studies which have used omics to assess GM crops.

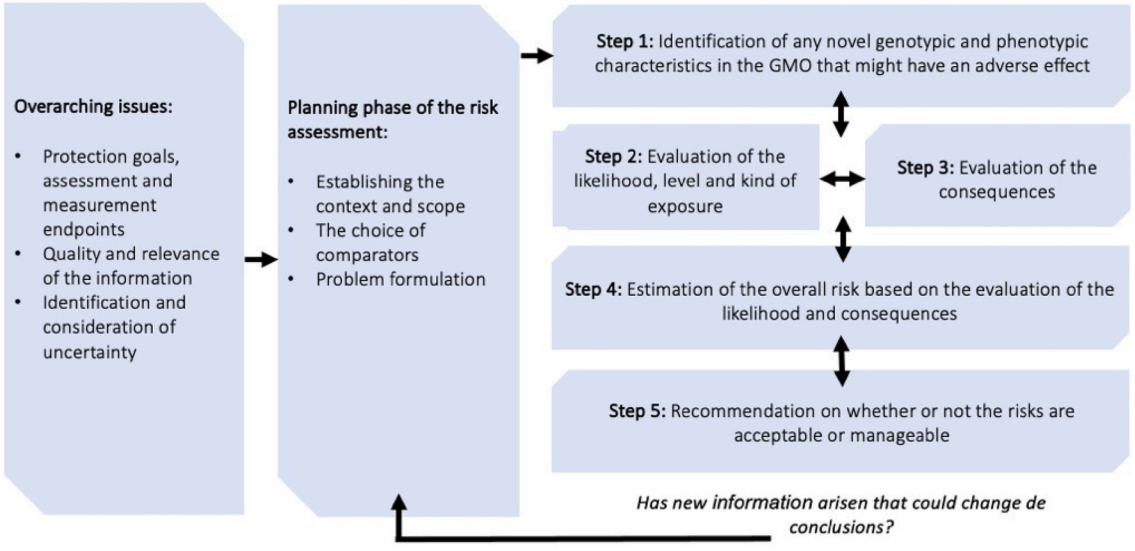

Fig. 1. Roadmap for risk assessment of genetically modified organisms. The flowchart illustrates a summary of the step-by-step risk assessment process proposed by the Ad Hoc Technical Expert Group (AHTEG) on Risk Assessment and Risk Management (UNEP 2014).

2. Approaches

2.1. Eligibility criteria, information sources and search strategy

We applied the following eligibility (inclusion and exclusion) criteria adapted from the structure of PICOS items (Supplementary file 1) (O'Connor et al., 2008, pp. 81–94). There was a language limitation to our review as only publications written in English were considered. Therefore, we acknowledge that this might be bias towards 'country' contributions. In addition, we excluded studies that were not published in peer-reviewed scientific journals. The period of our search was 2006–2020. All papers present in the databases from that period were included and analyzed.

The following databases were used: Agris (http://agris.fao.org), AGRICOLA (https://agricola.nal.usda.gov) and Scopus (https://www.scopus.com). For Web of Science (https://apps.webofknowledge.com), we have used the Core collection, SciELO and BIOSIS citation index. The search engines used were Google Scholar (https://scholar.google.no/) in which only the first 100 records were downloaded and JSTOR (jstor.org).

Preliminary searches were conducted to collect relevant publications relating to the research question. A list of keywords (search terms) was compiled from this cache of articles, which formed the base of the search strings. Synonyms were added, and terms adjusted to accommodate truncations, plurals and alternative spellings. Search terms were organized into three strings related to the inclusion criteria of the study. The first string relates to genetic modification, the second to omics-related techniques, and the third to genetically modified plants, largely based on the list of approved GM plants on the ISAAA list (Supplementary file 2). When the searches were performed, the strings were linked by the "AND" operator.

These strings, and combinations thereof, were modified according to the requirements of each database. Endnote version 9 (The EndNote TheEndNote Team, 2013) was used as a reference manager and repository for the records resulting from the searches.

2.2. Study selection and data collection process

A first search of the database yielded over 8000 records. At this stage, we introduced additional limitations to the inclusion criteria and decided to retain only studies that included *cry*, *epsps* and *bar/pat* transgenes. This was done to focus the review on commercially available (and most relevant) transgenes. It also had the effect of reducing the number of unique transgenes/events in the database since multiple studies done on the same transgene/event allowed better comparison between studies. After the removal of duplicates, over 3000 records remained in the database. A second round of screening followed, in which the abstracts were judged according to the inclusion criteria. If there was uncertainty about whether a publication should be included or not based on the abstract, title and keywords, judgement was made based on the full text. The final round of screening was done using the full text of each publication. The pipeline of our systematic review is presented in Fig. 2.

After study evaluation, the evidence for each outcome was synthesized separately for each study using a structured framework that contained five major information categories. Data collection was manually performed following each category established in the evidence table. The evidence table was built on Microsoft Excel (Excel version 16.41) by creating categories in which we could fit the extracted outcomes as well as study metrics and experimental design conditions. For each category, we have determined quantitative, qualitative, or descriptive classes that could cover, in general, the outcomes extracted from the papers

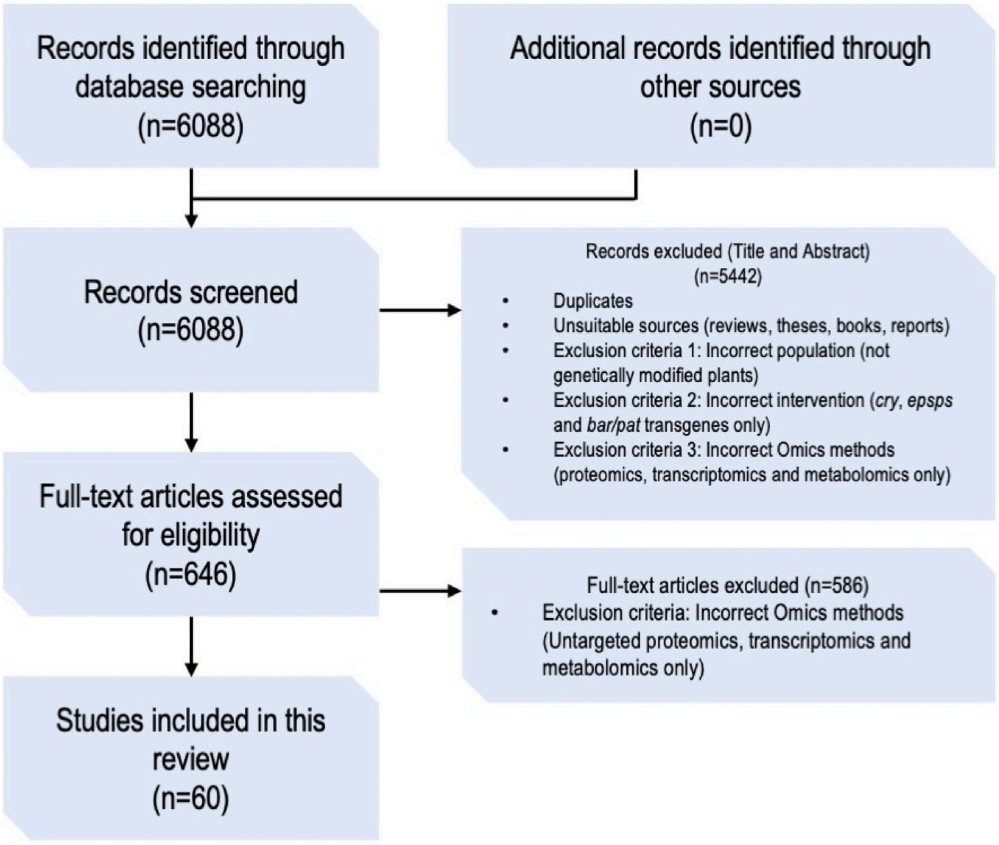

Fig. 2. Flowchart of assessment of eligible studies used in the systematic review and meta-analysis of metabolic effects in genetically modified crops assessed by omics studies.

(Supplementary file 3).

The classes used for transgenic techniques named "SDN1", "SDN2" and "SDN3" are described elsewhere (Agapito-Tenfen & Wikmark, 2015). The class "Not specified" was used whenever an item has been fulfilled, but information was not sufficient to add into the other specified classes. The class "None" was chosen whenever there was a complete absence of this item in the study.

For results on altered pathways of the included studies, the categories and classes in the evidence table were created based on the KEGG pathways database (Kyoto Encyclopedia of Genes and Genomes). When the authors did not perform a pathway enrichment analysis in their respective study, we manually searched the significant genes/proteins/metabolites against the KEGG pathway database and classified them according to their related pathway. Each pathway category included in the evidence table was defined following the KEGG pathways global categories under Metabolism ("Global and overview maps", "Carbohydrate metabolism", "Energy metabolism", "Lipid metabolism", "Nucleotide metabolism", "Amino acid metabolism", "Metabolism of other amino acids", "Glycan biosynthesis and metabolism", "Metabolism of cofactors and vitamins", "Metabolism of terpenoids and polyketides", "Biosynthesis of secondary metabolites", "Xenobiotics degradation and metabolism", "Chemical structure transformation maps"); Genetic Information Processing ("Transcription", "Translation", "Folding, sorting and degradation", "Replication and repair"); Environmental Information Processing ("Membrane transport", "Signal transduction", "Signaling molecules and interaction"); and Cellular Processes ("Transport and catabolism", "Cell growth and death", "Cellular community – eukaryotes", "Cellular community – prokaryotes", "Cell motility"). Classes under each category were defined as the specific pathways inside each global category according to the KEGG pathway database. Manually entered pathways were not tested for their significance.

2.3. Evidence synthesis and meta-analysis

To synthesize the data from the evidence table, we used the Excel program to produce panels with plots for what we considered the main categories. When available, informative mechanistic data were used to augment the qualitative syntheses. However, some data were synthesized using a narrative approach, with no meta-analysis performed due to the heterogeneity of endpoints and study designs considered in this review.

For the "Study metrics" category, the items of country, year, and authors affiliation were plotted into a map including the number of papers per country; a line plot showing the number of studies published per year; and a Venn diagram for information on authors affiliation. In the author affiliations category, "research institute" refers to an organisation that performs research independent of industry and public institutions (such as universities and government regulatory agencies). Descriptive statistics were also applied to the "Population" and "Intervention" categories. We have generated pie charts for the plant tissue sampled items, and a heatmap correlating the transgenes and plant species studied in the included publications was created. "Comparator" and "Study design" categories were synthesized using 2D column plots for all collected items. A Venn diagram was constructed to synthesize the omics platforms used in the reviewed studies. Finally, a meta-analysis on altered pathways was conducted and is presented as a heatmap of the global altered KEGG pathways in relation to the plant species and tissue sampled in the included studies. The heatmap value for each cell was calculated as the proportion (percentage) of the number of papers with the specific altered pathway, both positively and negatively, in relation to the total number of papers studying a particular plant species and sampled material.

Reporting of this systematic review follows the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al., 2009).

3. Results and findings

3.1. Study selection and evaluation

A total of 917 papers were retrieved through database searching and other sources, of which 352 proceeded in the next screening based on the titles and abstracts as full-text articles using the PICOS information. Of these, 277 articles were excluded in the second screening because they did not present endpoint assessment outcomes; were not transgenic plants; or presented improper data for the objective, such as other transgenes, target omics analyses, or no comparative statistical approach. Therefore, a total of 60 articles presenting unique experimental data published in the scientific literature were included in this review.

3.1.1. Study metrics

The studies included in this review were published between 2006 and 2020. Among these, the years with higher numbers of publications were 2015 (10) followed by 2012 (8) and 2008, 2018 and 2019 (6) (Fig. 3A). Trends in research affiliation have shown that the vast majority of the published studies (88.3%) were performed by universities (13) followed by research institutes (11) or collaborations between both types of institution (29) (Fig. 3B). The number of university-produced publications is in agreement with the higher proportion of studies funded by public sources (86.7%), and only three studies reported having industry support. The industry-funded studies were performed by industry researchers (1) or in cooperation with university and/or research institutes (2). These three studies were conducted by authors with affiliations in the U.S. Aside from a publication from 2008, all the contributions from the U.S. had affiliations with industry. There were also three studies conducted by regulatory agencies, all in collaboration with authors from universities and research institutes. Interestingly, only one study encompassed scholars from all four affiliation types. These studies can be found in evidence table using the filter tool which is provided in the Supplementary Information.

A geographical distribution analysis showed that authors affiliated with Chinese institutions produced the most papers (26), followed by Brazil (12) and Spain (8). Canada, India, Japan, Finland, Sweden, Estonia, and Saudi Arabia had only one study published (Fig. 3C). The publication timeline for China and Brazil was consistent and suggests long-term public funding for the topic.

3.1.2. Evaluation of plant species and transgenic inserts

There were no exclusion criteria for plant species in our search strategy. However, due to the restriction on transgenes commonly found in transgenic crops, the selected studies were performed in only seven plant species, out of which five are crop commodities (i.e. oilseed rape, maize, cotton, soybean and rice). The two non-commodity species were the poplar (*Populus* sp.) (1) and the well-known model species Arabidopsis (2). Except for the studies using Arabidopsis and poplar, most of the studies analyzed commercially available crop varieties. This is also demonstrated by the large number of publications with maize varieties containing the *cry* transgenes (23) (also known as Bt maize) and soybean varieties containing the *epsps* transgenes (13) (also known as Roundup Ready soybean). Transgenic crop varieties carrying these transgenic events are still the most commercialized varieties worldwide (ISAAA, 2018) (Fig. 4B).

The most analyzed plant materials were seed/grain (29) and leaf samples (26), accounting for 91.6% of the studies reviewed (Fig. 4A). Although these two plant materials have clear safety interest, either for food and feed consumption or potential environmental impact, researchers were also interested in analyzing other samples for different testing hypotheses. Soybean, maize and rice embryos were analyzed by three studies, as were rice seedlings (3), and stems (2).

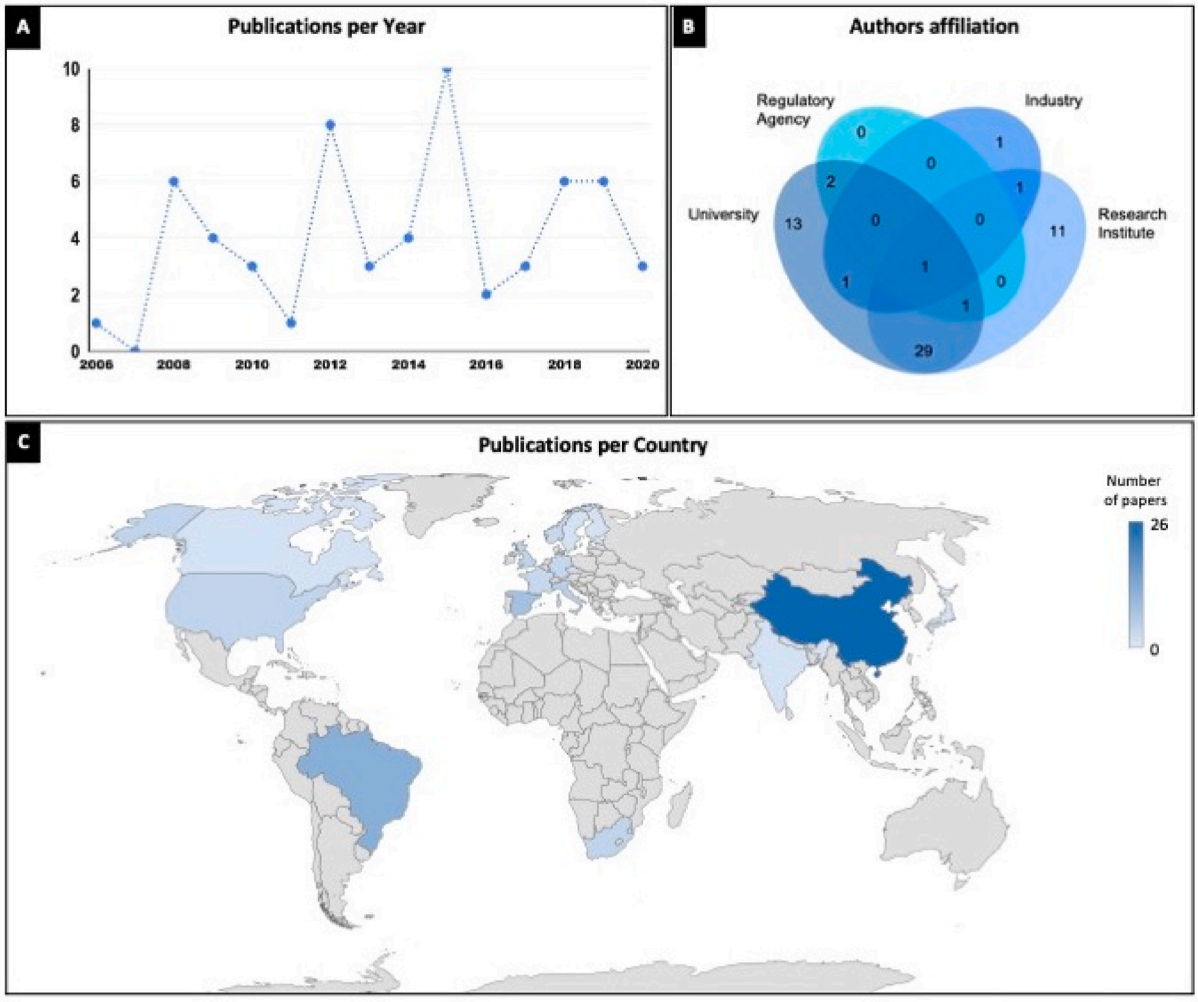

Fig. 3. Number of publications on alterations in genetically modified crops assessed by omics studies from 2006 to the present. The data were extracted from several databases, including the Web of Science Core Collection, by August 2020 by searching publications containing the PICOS information pertaining to the systematic review research question. (A) Number of publications per year; (B) Authors affiliation; and (C) Number of publications per country.

3.1.3. Evaluation of experimental designs and statistical approaches

We have analyzed aspects of the experimental design, such as the growing conditions, number of biological and technical replicates and type of comparator and controls used. In addition, we have carefully analyzed whether abiotic and biotic stressors were applied and what statistical approach was used.

With regards to the experimental growing conditions, 40% (24) of the studies had field growth experiments; 26.7% (16) had experiments conducted in growth chambers; and 18.3% (11) under greenhouse conditions. Six studies used in vitro tissue-based samples in their methodology, and four studies either did not specify the experimental growing conditions (3) or used an alternative setting method (1) (Fig. 5A). Most did not evaluate samples across different field or growing seasons, as only one sample collection was performed. This was also observed for greenhouse and growth chamber experiments. The number of individual plants used per sample was variable and ranged from one up to 100 plants. Surprisingly, half of the studies (31) did not specify this in their reports. The number of replicates, either biological or technical, also varied among the selected studies. More than three biological replicates were used in approximately half of the studies (33), whereas almost one third did not specify how many replicates were used. With regards to the technical replicates used for the different platforms, it was observed that 40% of the studies used three or more technical replicates, 11.7% used fewer than three replicates, and 43.3% did not report how many technical replicates were used. There was no correlation between the omics platform and the number of replicates used. A synthesis of information on comparator type used showed that most studies used a non-GM near-isogenic line as the control (37). Other studies did not apply the near-isogenic variety as a comparator but used the parental line (13), the reference variety (8), or the wild type (7) instead. There was one publication in which the comparator used was not clear in the methods (Fig. 5B).

The application of stress conditions in the experimental approach in the selected studies was not a trend, as approximately 80% did not apply any stressor conditions. Four out of 60 studies investigated the effects on GM plants when herbicide was applied. These are: Benevenuto et al., 2017, Bernillon et al., 2018, Mesnage et al., 2016, Zanatta et al., 2020; and two studies applied drought stress, these are Benevenuto et al., 2017 and Gulli et al., 2015.

A range of transcriptomics, proteomics and metabolomics techniques were applied in studies of GM crops in recent years. Sixteen out of 60 studies used methods in transcriptomics (i.e., microarray or RNA-seq) alone (11), or in combination with other omics tools (5), to study gene expression in GM crop lines and their non-GM comparators. In all transcriptomic studies, the authors found statistically significant differences in the expression of transcripts between GM and non-GM comparator. Gel-based and gel-free proteomics techniques were used in 27 of the publications reviewed, of which four applied proteomics in

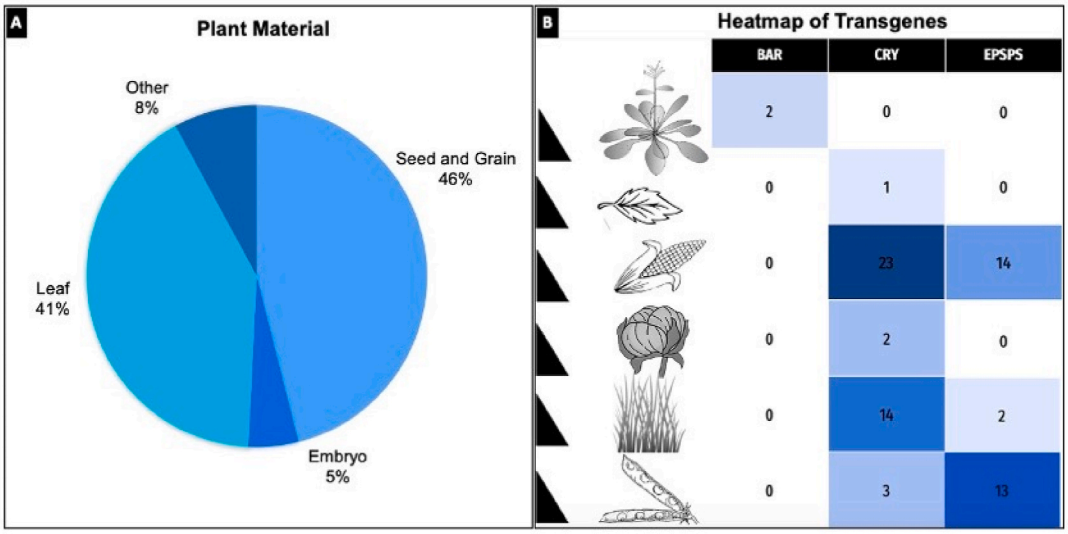

Fig. 4. Number of publications on alterations in genetically modified crops assessed by omics studies from 2006 to the present. The data were extracted from several databases, including the Web of Science Core Collection by August 2020 by searching publications containing the PICOS information pertaining to the systematic review research question. (A) Plant material sampled; (B) Heatmap of transgenes and plant species studied.
Note: The plant species analyzed were (from top to bottom): *Arabidopsis thaliana* (arabidopsis), *Brassica napus* (canola), *Zea mays* (maize), *Gossypium mustelinum* (cotton), *Oryza sativa* (rice) and *Glycine* max (soybean).

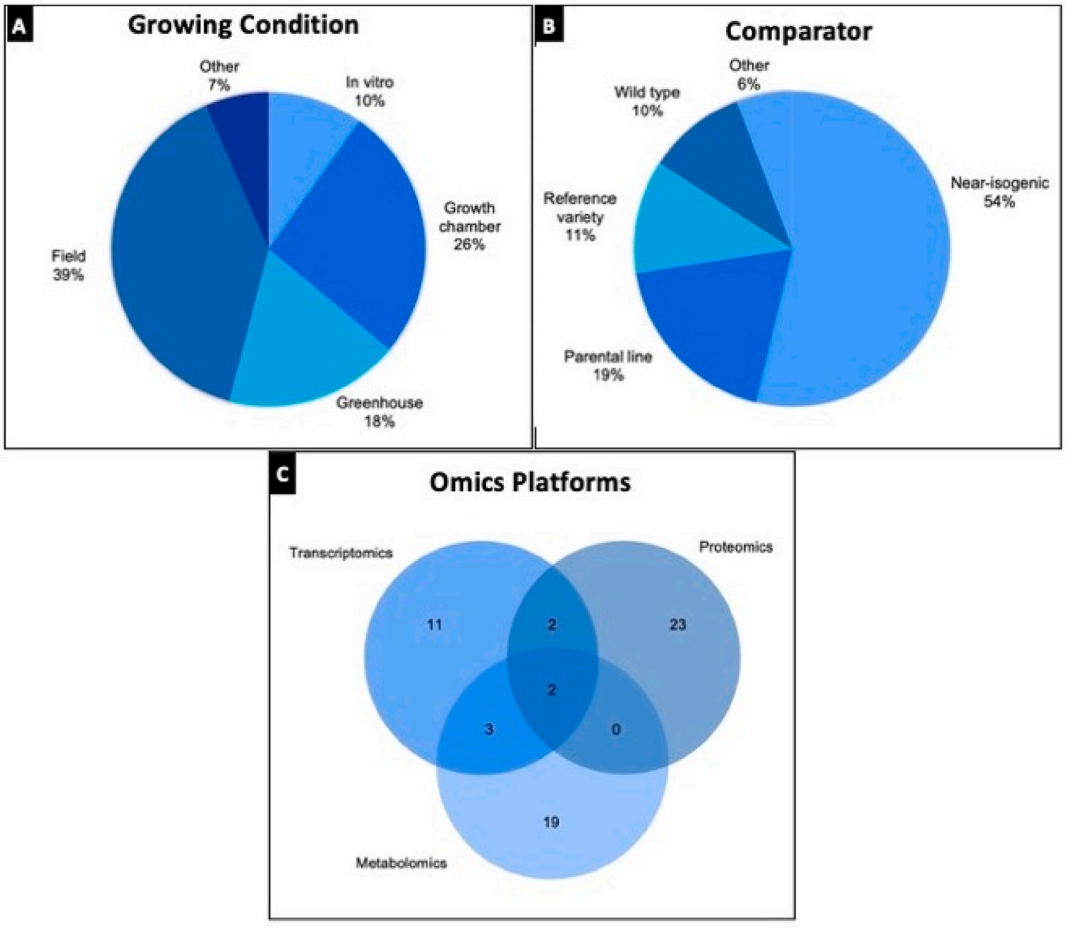

Fig. 5. Number of publications on metabolic effects in genetically modified crops assessed by omics studies from 2006 to the present. The data were extracted from several databases, including the Web of Science Core Collection by August 2020 by searching publications containing the PICOS information pertaining to the systematic review research question. (A) Experimental growing condition; (B) Comparator/control type. (C) Omics platform used in the reviewed studies.

combination with metabolomics (2) and transcriptomics analyses (2). All studies that conducted a comparative proteomic profiling analysis found at least one protein with a statistically significant difference in abundance between the GM line and its control (Supplementary file 4). Lastly, metabolomics was used in 26 studies, alone (19) or in conjunction with proteomics and/or transcriptomics techniques (7) (Fig. 5C).

Our review of statistical methods showed that only three studies, all based on metabolomics platform, did not run a comparative analysis. Most studies applied inferential statistics based on a *t*-test (50), of which in half (25) applied a fold change as a complementary cut-off parameter. The remaining studies used only fold change (2), presence-absence (2) or an alternative cut-off parameter (6). Thirty-five percent of the included omics studies did not conduct any descriptive analysis of their data. The studies that applied a descriptive statistic opted mostly for the classical Principal Component Analysis-PCA (28); Heat maps (9); or Volcano plots (5). In terms of validation, only 28.3% of the reviewed studies applied a validation method of the results found through omics techniques. Validation methods used were PCR (15), mostly for transcriptomic studies, blotting techniques (3), or other alternative methods (2). Surprisingly, we found that only 19 out of 60 studies conducted any type of multiple comparison correction test on the obtained results (Supplementary file 4).

3.2. Study outcomes and meta-analysis of altered pathways

We have conducted a meta-analysis of the altered genes, proteins and metabolites reported in the included studies. A heatmap network meta-analysis has been used to visualize and compare multiple transgenic events in a single analysis simultaneously. Further statistical analyses could not be performed due to the high heterogeneity of the studies.

The heatmap analysis showed that the Kegg-defined metabolic pathways most often reported to be altered were those involved in the metabolism of carbohydrates, energy, lipids, and amino acids, as well as genetic information processing (GIP) and environmental information processing (EIP) of signal transduction (Fig. 6); (Supplementary file 5). On the other hand, metabolism of glycan and chemical structure transformation and cellular processes (CP) of cell motility were the least reported pathways in the reviewed studies.

EPSPS-expressing maize leaf and seed profile showed similar patterns, with most metabolic alterations seen on biochemical pathways related to carbohydrate metabolism and biosynthesis of secondary metabolites. However, alterations to pathways relating to amino acid metabolim and genetic information processing were most present when leaf tissue was studied. For maize expressing Cry proteins (Bt toxins), pathways were similarly affected; with strong hits for carbohydrate and amino acid metabolism, as well as biosynthesis of secondary metabolites. For EPSPS-expressing soybean samples, the same major pathways were affected (carbohydrate, amino acid and the metabolism of biosynthesis of secondary metabolites), however, not as pronounced as in maize samples.

We observed that studies that analyzed leaf tissue of dicotyledonous species (soybean, cotton, and arabidopsis), regardless of the transgene, did not show altered lipid metabolism pathways. However, the only publication on EPSPS transgene of rice seed showed lipid and amino acid metabolism, GIP, and CP of cell growth and death as the pathways affected. Cry transgenic rice studies also showed a variety of metabolic pathways, GIP, and EIP pathways being altered, though pathways involved in metabolism of terpenoids and other secondary metabolites, and EIP of signaling molecules which were exclusively found in rice leaf studies, while metabolism of chemical structure transformation and CP of cell growth and death were only reported when using seeds.

The two omics studies on Cry transgenic cotton leaves reviewed here also showed pathways relating to metabolism (especially energy metabolism) were affected. GIP, EIP of signal transduction, and CP of transport and catabolism were also found in at least one of these studies. Finally, following the general trend, studies of BAR transgene on arabidopsis leaf found metabolism of carbohydrates, energy, amino acid, as well as GIP as the altered pathways. The complete list of altered pathways is provided in Supplementary file 5.

Fig. 6. Pathway-level heatmap depicts metabolic pathways whose expression was significantly altered in genetically modifies plants containing *cry* and *epsps* transgenes and the tissues assessed by plant species. Heatmaps clusters are provided for all KEGG-defined pathways. This view illustrates that the most significantly altered KEGG-defined pathways fall into several key areas of primary metabolism.

4. Discussion

4.1. Identification of metabolic changes in genetically modified plants

Whereas omics technologies also include the analysis of DNA sequences, such as genomics, this study focused on particular omics approaches from the transcriptome onward and we did not cover other relevant omics.

Several altered metabolic pathways have been found in the comparative omics studies assessing alterations in GM crops. Carbohydrate and energy metabolism were the most frequently altered pathways for all three transgenes (*epsps*, *cry*, and *bar*). The reason for this might lie in the current strategy for transgenic expression, which is based on strong viral constitutive promoters (e.g. P35S). In such cases, downstream transgenes are largely expressed in all organs and at all stages of the plant's life cycle. The expression of transgenes at some concenbtration or at any level in particular tissues could cause metabolic changes in the organism The constitutive overexpression of a transgene may compete for energy and building blocks to synthesize proteins, RNA, and metabolites required for plant growth under normal conditions (Singhal et al., 2016).

Such energetic impact can also escalate as it directly affects metabolic cascades responsible for integrated and sensitive responses to multiple signals. This has been seen in constitutive expression of signal-transduction promoters for pathogen resistance that have led to a decreased growth and enhanced susceptibility to ~~other~~ pathogens (Berrocal-Lobo, Molina, and Solano 2002; Bowling et al., 1997). When constitutive promoters were replaced by stress-inducible overexpression of the transcription factor AtDREB1A in transgenic Arabidopsis and rice, it showed enhanced tolerance to chilling, drought and salt stress with overcoming the problem of growth retardation (Kasuga et al., 2004). Similar results were observed in potato (*Solanum tuberosum*), *Dendranthema grandiflorum* and peanut (*Arachis hypogea*) by Hong et al. (2006); Behnam et al. (2007); and Bhatnagar-Mathur et al. (2007). Abnormal leaf morphology, such as twisted and bending edge and severely wrinkled leaf shape, has also been observed in the constitutive overexpression of the XTH gene from hot pepper in transgenic Arabidopsis with improved tolerance to severe water deficit (Cho et al., 2006).

Whereas the constitutive expression of a transgene may provide a desirable trait; this positive effect may provoke other adverse physiological effects in the plant. Various studies have revealed that a constitutive expression of a transgene causes either stunted/abnormal growth under normal conditions or mild/severe growth retardation in the aerial parts and reduced sugar content, compared to non-transformed plants (a review can be found in Singhal et al., 2016). It has been observed for salt-treated transgenic tomato plants overexpressing the HAL1 transgene, whose function on sodium homeostasis is very clear, that it had pleiotropic effects on osmotic homeostasis. Thus, the metabolic or energy costs may mask and limit the benefit of a transgene, resulting in growth and yield penalty. The transgenic line overexpressing HAL1, with a very high exclusion capacity of sodium from the leaves, appear to be an energy costly ability resulting in reductions in fruit yield (Muñoz-Mayor et al., 2008; Rus et al., 2001).

Pleiotropic effects or any other adverse effect from the constitutive expression of a transgene can lead to a pathway to risk. Whereas growth penalty might only have agronomic impact, sensitivity to stress as a pleiotropic effect can lead to the production of toxins in the plant (Prescott et al., 2005; Zolla et al., 2008; Graf et al., 2014). Transcriptomic analysis of cry1ab transgenic maize (MON810 event) under drought conditions showed large stress-related metabolic alterations when compared to its conventional maize counterpart (Gullì et al., 2015).

In this review, we have considered all statistically significant results as indicators of potential metabolic alterations in the GMO. However, the authors performing the studies have not always drawn the same conclusion as us that the statistically significant differences should be considered to be metabolic changes in the GMO. For example, Zhu et al. (2008) and Gulli et al. (2015) compared the gene expression profiles of GM soybean and maize, respectively, following transcriptomic microarray analysis. In both studies, the authors used a non-GM near-isogenic variety for the comparative analysis and found statistically significant differences in the expression of certain genes. However, the authors state that such differences are "minor" or "at or near the empirical false discovery rate" and, therefore, not biologically significant. Zhu et al. (2008) tested the potential secondary effects of glyphosate on soybean, and also differences in gene expression between cotyledons of GM glyphosate-resistant and non-GM glyphosate-sensitive plants. They found 18 genes significantly affected in GM glyphosate-resistant plants 1–24 h after glyphosate application and 2 other differentially expressed genes when comparing gene expression profiles of GM and non-GM cotyledons without any glyphosate treatment. Despite these results, the authors concluded that there are few unexpected changes in the transcriptome associated with the use of GM glyphosate-resistant soybean in agriculture, but this should not raise a concern. Gulli et al. (2015) compared the gene expression profiles of GM maize and a non-GM near-isogenic comparator under drought and optimal growing conditions in the field. The authors observed a greater number of differentially expressed genes in the non-GM variety compared to the GM under drought stress. These were mostly genes coding for heat shock proteins, late embryogenesis abundant proteins, and detoxification enzymes, which are considered key genes for a more efficient response to drought. Although these results clearly indicate a different stress response pattern in the GM variety compared to its non-GM comparator, the authors concluded that these results could not be considered substantial since the global gene expression pattern under controlled growing conditions was similar. Other studies similarly concluded that genetic modification caused less variation than natural variability derived from conventional breeding and genotypic variation (Clarke et al., 2013; Coll et al., 2010; Harrigan et al., 2010; Rao et al., 2016).

Some metabolomics studies have used alternative experimental and statistical methods rather than conventional comparison between GM and non-GM near-isogenic lines commonly used for comparative omics data analysis. For instance, Kusano et al. (2014) assessed the metabolomic diversity of a soybean lineage representing 35 years of breeding, including the analysis of seeds from 3 GM to 6 non-GM conventional lines. Based on multivariate exploratory and discriminant analysis (Principal Component Analysis-PCA and Orthogonal Projections to Latent Structures Discriminant Analysis-OPLSDA) and equivalence testing, the authors suggested there were no clear metabolic differences between the GM and conventional lines. Equivalence testing is a relatively recent approach for omics data, where a set of reference conventional varieties is used to generate a range of abundance values for each metabolite, and corresponding values from a GM variety are assessed to determine if they are within this reference range. EFSA recognizes this approach in their guidance document (EFSA, 2011), but equivalence testing is regarded as a complementary test and must be combined with the classical difference test between the GM line and its non-GM near-isogenic comparator. While the test of difference is used to verify whether the GM plant, apart from the genetic modification, is different from its comparator and has the potential to cause alterations, the test of equivalence is used to understand the biological relevance of differences found. In Kusano et al. (2014), the authors applied the equivalence test but did not conduct difference tests comparing the metabolite profiles of GM lines against those of their appropriate non-GM comparators. Another metabolomic study applied non-targeted metabolomic analysis to characterize GM and conventional maize varieties (Václavík et al., 2013). The authors concluded based on descriptive statistics (i.e. PCA) that both varieties are substantially equivalent because the variability of the metabolites in the GM line did not exceed the ranges measured within the conventional lines. However, no comparative test of difference between the metabolic profiles was performed (Václavík et al., 2013). Harrigan et al. (2010) is another

similar case where the authors work with multivariate descriptive (Principal Variance Component Analysis – PVCA) and correlation (Canonical Discriminant Analysis – CDA) analyses in seed metabolite profiles of GM and non-GM soybean. Although they found the major source of metabolite variability associated with variety factor, the authors state that transgenic and conventional lines were not uniquely distinguishable from each other, supporting the theory that genetic modification is not a meaningful contributor to metabolite variability (Harrigan et al., 2010).

The preceding years have shown that carrying out the environmental risk assessment of GMOs without a definition of biologically relevant effects and of environmental harm led to substantial controversies about their environmental safety (Dolezel et al., 2017; AHTEG 2014). Dolezel et al. (2017) have shown that the underlying controversy on GMO risk assessment often derived from a different perception of what constitutes environmental harm. While science can support decisions on the relevance of adverse effects observed, it cannot make any of the normative decisions on what, where and when to protect. Similar controversy can be anticipated for omics data in risk assessment in which experts might disagree on what metabolic changes can lead to 'harm'. Therefore, we consider it necessary to define biological relevant changes in omics outcomes which may be used as a prediction of harm for a specific protection goal. Further clarifications will also be needed if omics comparative assessments are to be used for defining environmental 'limits of concern' parameters.

4.2. Variability of omics methods and the need for harmonization

Investigating GMO metabolic changes is a complex task, and the lack of harmonization of analytical methods makes it more complicated. So, while the utility of applying omics techniques such as sequencing analysis and mass spectroscopy to the task of generating data concerning metabolic alterations is clear, the wide range of potential techniques makes the GMO risk assessment a very complex decision-making procedure.

The lack of consistent reporting on the experimental design and methodologies of the studies included in this review added an extra challenge to the analysis of the experiments. Despite many efforts from the academic community to establish minimum reporting requirements for the different omics techniques, the majority of the studies did not include basic information on the experiments. There have been large initiatives for developing guidelines for several omics techniques in the past, such as microarrays (Brazma et al., 2001), proteomics experiments (Taylor et al., 2007), plant metabolomics studies (Fiehn et al., 2007), as well as for quantitative real-time PCR experiments (Bustin et al., 2009). More recently, other groups have developed a generic transcriptomics reporting framework (TRF) for omics data processing and analysis (Gant et al., 2017). These initiatives provide frameworks for the standardization of reporting of omics data generation and analysis to ensure that all of the information is available to understand, interpret and reproduce an omics experiment and its results. These frameworks build a good foundation for ensuring that sufficient information is available to evaluate the quality of the experimental data and interpretation and support reproducibility.

Of particular concern is that only 61.6% of the studies used the non-GM near-isogenic variety as a control or comparator. The other 38.4% used parental lines, reference varieties, wild types, and other comparators as control. Although the use of alternative comparators can be valuable, the use of the near-isogenic variety is considered the most appropriate compataor in risk assessment procedures in Europe and elsewhere (EFSA, 2008; CBD, 2003). Therefore, it is clear that adaptation from these frameworks should be developed not only to provide sufficient information but also to meet standards and regulatory compliance for GMO risk assessment.

Technological development of omics methods has quickly evolved in the last two decades. Omics profile analysis can identify combinatorial effects due to interactions between transcripts, proteins, and metabolites produced by genes, or the interaction between them. However, changes in transcriptomes, proteomes, and metabolomes are affected not only by genetic factors, including genetic modification, but also by a range of internal and external factors (Li et al., 2017). Therefore, the range of different experimental setups needs to be considered when developing harmonized methods.

Notably, most of the transcriptomic studies included in this review applied microarray technology (11 out of 16 studies). In general terms, microarrays quantify a set of predetermined sequences, called "probes", through fluorescence intensity. Although this is an efficient technology for answering many research questions, the technique has several technical limitations regarding sensitivity and the ability to reliably detect all possible alternatively spliced transcripts (Davies, 2010). In addition, microarrays require prior knowledge on annotated reference transcripts of the organism of interest (Mantione et al., 2014). The newest transcriptomic studies included in our review were based on a different transcriptomic technique, high-throughput RNA-sequencing (RNA-seq). RNA-seq is an omics technique in which the cDNA transcripts are sequenced, and the abundance is derived from the number of counts of each sequenced transcript (Lowe et al., 2017). The main advantages of this technique are the higher sensitivity and dynamic range and the capacity to detect novel sequences through *de novo* assembly methods (Lowe et al., 2017; Mantione et al., 2014; Wang et al., 2009). Both transcriptome profiling techniques can be used to investigate changes in gene expression of new GM crop, and have the potential to detect metabolic effects (Chassy, 2010; Gong & Wang, 2013).

Proteomic methods are also widely used in the safety assessment of GM crops. Among the studies included in this review, two non-targeted proteomic profiling approaches were employed: gel-based and gel-free techniques. The vast majority of proteomic studies reviewed here used gel-based techniques (21 out of 27), while only six worked with gel-free methods. Gel-based proteomic profiling techniques include the traditional two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and a more recent version called two-dimensional difference gel electrophoresis (2D-DIGE), which uses differential labelling of protein samples with fluorescent tags, offering greater sensitivity and reproducibility for differential quantitative analysis of protein expression (Meleady, 2018). Both gel-based methods must be used in conjunction with a mass spectrometry technique (i.e., MALDI-MS or tandem MS/MS) to identify the proteins. We notice that most papers using gel-based techniques in this review were published between 2008 and 2017, while more recent studies (2018 onwards) tended to use gel-free methods. Gel-free proteomics techniques based on liquid chromatography have become more popular in recent years, although it still requires considerable investment in expensive mass spectrometry instruments and highly skilled personnel to run the facility. Advanced gel-free systems provide more sensitive and accurate protein separation and quantification and are less time-consuming and labour-intensive (Jain et al., 2019). Regardless of the method chosen, all 27 papers presenting comparative proteomic profiling identified statistically significant differences between GMO and non-GM plants. This indicates that proteomics techniques are powerful tools in detecting metabolic effects caused by genetic modification in GM crops.

In the last decade, metabolomic studies for risk assessment of GM crops have become more prevalent. Our review showed that most metabolomic studies on GM plants were conducted in the past decade. Because untargeted metabolomics can analyze all detectable metabolites in a given sample simultaneously, this approach is a promising replacement for the conventional compositional analyses that were limited to a restricted set of analytes for safety assessment (Christ et al., 2018). In recent years the field of metabolomics has witnessed significant advances in both instrumentation and software development (Wolfender et al., 2015), but metabolite identification is still a major bottleneck in untargeted metabolomics. Although over 200 000 plant metabolites are known (Wurtzel & Kutchan, 2016), only a small fraction

can be annotated using databases (Christ et al., 2018). The metabolomics research community is currently working hard to address challenges regarding standardization of the methods, statistical considerations, and annotation of metabolites (Spicer, 2017b; 2017a). Increasing the annotation of plant metabolites in public databases will result in a more robust and replicable technique in the context of risk assessment of GM crops. We also notice that data analysis is still challenging, as analysis results in a vast metabolomic dataset require the combination of multiple statistical approaches, from descriptive (e.g., PCA and hierarchical clustering) to inferential (e.g., analysis of variance) statistics.

Some authors have discussed how the integration of multi-omics approaches could substantially contribute to a comprehensive understanding of potential metabolic changes of GM crops, covering all major classes of biomolecules (Christ et al., 2018; Li et al., 2017; Heinemann et al., 2011). While studies using a single omics approach have been reported on different inserts and plant species, comprehensive system biology analyses of GM crops at all levels are still scarce. In this review, we found seven out of 60 published papers, which combined more than one omics platform in their study. Only two of them combined transcriptomic, proteomic, and metabolomic methods in their analyses. This is understandable since integrating omics platforms in a single study is not easy, particularly because of the high cost and advanced structure required and because it is possible to publish each omic as a stand alone paper. Besides, there are still challenges shared among the omics technologies that must be taken into consideration when projecting a multi-omics study: handling large datasets (e.g. filtering and cleaning, transformation, normalization, and scaling); annotation of biomolecules in public databases; study design and analytic assumptions; statistical power (e.g. sample size vs number of biomolecules quantified); data archiving and sharing (e.g. lack of standardized nomenclature, data formatting, and public access to datasets) (Misra et al., 2019).

With the rapid progress of omics technologies, the scientific community needs to embrace the methodological challenges, as well as commercial developers, and work in establishing consistent protocols, such as standardized sample quality, sample and data analysis pipelines, as well as data formats for public data availability (Misra et al., 2019). Furthermore, a dedicated framework must be developed to meet GMO risk assessment regulatory demands.

4.3. Towards an implementation pathway for omics in GMO risk assessment

The OECD in 1993 developed the principle of substantial equivalence, guidelines and recommendations for risk assessment of GM crops that many countries have built upon. This principle is based on the concept that a near-isogenic conventional variety, with a history of safe use, can serve as a comparator when assessing the safety of a novel GM variety. However, the current approach has been criticized because it is not effectively designed to detect metabolic changes that may arise from the genetic transformation process (Catchpole et al., 2005; Ladics et al., 2015; Levidow et al., 2007; Picone et al., 2011).

The current safety assessment procedures include a compositional analysis of the GM crops which is primarily based on a targeted analysis by looking at specific key metabolites (e.g., nutrients and antinutrients, allergens, proximates, etc. However, the biological relevance of these data, or at least their value in predicting harmful events, is not clear. Because of the great potential for detecting effects at all levels, some studies have advocated for the inclusion of untargeted high-throughput omics techniques for the future assessment approach of new biotech crops (Christ et al., 2018; Heinemann et al., 2011; Howell et al., 2018; Kok & Kuiper, 2003; Li et al., 2017; Pielaat et al., 2013). In contrast, industry scholars have disagreed with the need for a new risk assessment framework based on including untargeted omics approaches (Raybould et al., 2019). Recently, a letter from industry researchers (Delaney et al., 2019) criticized a published study where the authors propose incorporating a metabolomic-centered framework to improve the risk assessment of biotech crops (Christ et al., 2018). The authors state that GM crops are currently adequately tested and subjected to an extensive molecular characterization, which includes the analysis of approximately 70 analytes (Delaney et al., 2019).

On the other hand, EFSA has recently organized a scientific colloquium on omics in risk assessment to explore the opportunities for integrating datasets produced via specific omics tools within risk assessment approaches. The report concluded that omics technologies are a valuable addition to risk assessment of food and feed products and the environment. It also pointed to a need for a consistent reporting framework for data collection, processing, interpretation, storage and curation, which should be further drawn up together with national and international organisations towards its routine use in risk assessment. The authority also suggested the use of test cases that could be worked out to enhance confidence in the use of omics datasets in risk assessment (EFSA, 2018). In line with EFSA, the U.S. National Academies of Sciences, Engineering, and Medicine also reports on the usefulness of omics technologies to enable an examination of a plant's DNA sequence, gene expression, and molecular composition. These techniques are expected to improve non-GM and GM crop development efficiency and could be used to analyze new crop varieties to test for metabolic changes caused by genetic engineering or conventional breeding (Ning et al., 2018).

The idea behind these institutional efforts is to introduce omics technologies in the risk assessment framework, not to replace the entire existing analyses, but to improve the existing approach, for instance, through the validation and supplementation of the data. Also, the moment for updating and incorporating more holistic analysis on risk assessment of GM crops comes as products developed with new genetic engineering tools, like CRISPR/Cas, are being developed by the industry.

The major challenge for efficient omics implementation then relies on an old problem – the definition of biological relevance of the generated data (Fig. 7).

In addition, in order for omics to be implemented in GM crop risk assessment, as analyzed in this review, there is a need for a harmonization process of validated omics methods in all analysis steps and the establishment of a user-friendly multi-omics framework of GM crops risk assessment. The establishment of a standardized protocol for the assessment of GM crops based on a multi-omics approach would help foster a more comprehensive understanding of potential metabolic effects at all biological system levels. For instance, guidance on the selection of appropriate comparator(s), known as non-GM near-isogenic counterparts, is a regulatory prerequisite for good experimental design (EFSA, 2011). Also, for plants with stress tolerance, they should be cultivated under different growth conditions (e.g., field and greenhouse), at multiple geographical locations, and with the treatment designed for the new trait (e.g., complementary herbicide applications). The new multi-tiered framework must also provide user-friendly pipelines for sample analysis, as well as data analysis with the available statistical tools and interpretation of results. Finally, integration of untargeted multiomics approaches, and the combination with some targeted analyses, could bring more sensitivity for detecting a larger range of metabolic effects. For that, recent approaches and existing tools have been discussed for the development of standardized analytical pipelines that could be adopted by multiomics projects (Misra et al., 2019). For instance, descriptive and exploratory approaches under multivariate analysis (i.e., principal component analysis – PCA; and canonical correlation analysis - CCA) can reduce data dimensionality and correlate the variables. Also, a range of analytical tools (Eicher et al., 2020), analysis workflows (Lancaster et al., 2020), as well as enrichment analysis tools for pathway- or biological-network-based in integrative multiomics (Wanichthanarak et al., 2015) are available.

5. Conclusions

This systematic review revealed a clear lack of detailed information

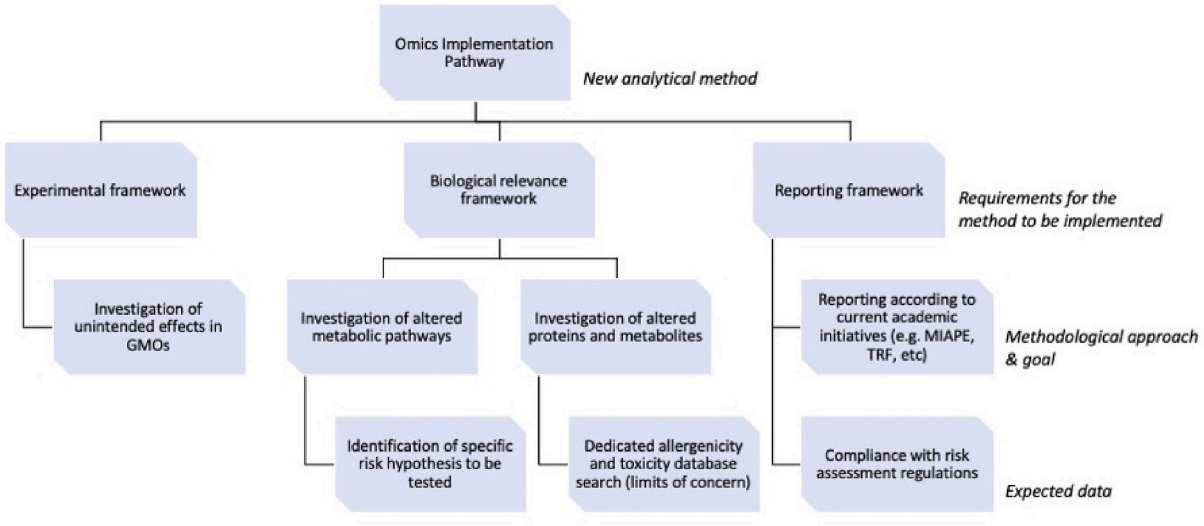

Fig. 7. Implementation pathway for omics into GMO risk assessment. Major areas for framework development in order to achieve regulatory demands.

on experimental designs in the publications examined, which posed a limitation to our meta-analysis. In the context of altered metabolic pathways, we found carbohydrate and energy metabolism as the most affected pathways, which corroborates the concerns raised over potential fitness cost that expression of transgenic proteins could cause. The literature shows that fitness cost may lead to pronounced stress metabolism due to potential adverse effects on the plant, human health, or the environment.

In addition, no consensus exists regarding experimental design, choice of comparator, use of appropriate omics technique, statistical analysis, and biological relevance of the results. Therefore, for efficient regulatory implementation, there is a need to develop frameworks related to proper reporting, dedicated experimental setups, and a framework for the definition of biological relevance of the generated data. Nevertheless, the lack of harmonized methods seems to be due to the rapid progress of omics technologies rather than inconsistent reporting. Such lack of harmonization hampers a broader understanding of the effects of genetic modifications in different plant species and traits. Therefore, important considerations need to be made for the design of future successful studies, for instance, the integration of multiomics platforms; validation of results; contributions for public databases; as well as the development of guidelines with standardized and user-friendly frameworks.

We conclude that omics techniques are suitable tools to comprehensively screen for alterations in genetically modified plants. In light of the speed of development of new GMOs, new tools such as omics are needed to enable a comprehensive risk assessment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tifs.2022.01.002.

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