MS-BASED ANALYTICAL METHODOLOGIES TO CHARACTERIZE GENETICALLY MODIFIED CROPS

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ABSTRACT

The development of genetically modified crops has had a great impact on the agriculture and food industries. However, the development of any genetically modified organism (GMO) requires the application of analytical procedures to confirm the equivalence of the GMO compared to its isogenic non-transgenic counterpart. Moreover, the use of GMOs in foods and agriculture faces numerous criticisms from consumers and ecological organizations that have led some countries to regulate their production, growth, and commercialization. These regulations have brought about the need of new and more powerful analytical methods to face the complexity of this topic. In this regard, MS-based technologies are increasingly used for GMOs analysis to provide very useful information on GMO composition (e.g., metabolites, proteins). This review focuses on the MS-based analytical methodologies used to characterize genetically modified crops (also called transgenic crops). First, an overview on genetically modified crops development is provided, together with the main difficulties of their analysis. Next, the different MS-based analytical approaches applied to characterize GM crops are critically discussed, and include "-omics" approaches and target-based approaches. These methodologies allow the study of intended and unintended effects that result from the genetic transformation. This information is considered to be essential to corroborate (or not) the equivalence of the GM crop with its isogenic non-transgenic counterpart.

I. INTRODUCTION.

A. DNA recombinant technology.

Since its introduction in the 70s, recombinant DNA technology (or genetic engineering) has become one of the foremost technological advances in modern biotechnology. Genetic engineering allows selected individual genes to be transferred from an organism into another and also between non-related species. The organisms derived from recombinant DNA technology are termed genetically modified organisms (GMOs), and are defined as those organisms in which the genetic material has been altered in a way that does not occur naturally by mating or natural recombination (WHO, 2002). Apart from recombinant DNA technology, other techniques that fall under the GMO definition include methods for the direct introduction of DNA and cell or protoplast fusion techniques, whereas in-vitro fertilization, natural transformation and polyploidy induction are excluded from the definition (Kok *et al.*, 2008).

In plant biology, recombinant DNA technology has become an indispensable tool for the experimental investigation of many aspects of plant physiology and biochemistry that cannot be addressed easily with any other experimental means (Wisniewski *et al.*, 2002). Thus, recombinant DNA technology offers an unprecedented opportunity to study the molecular basis of important processes, such as development, plant-microbe interactions, response to abiotic and biotic stress, and signal transduction pathways, by the analysis of gene function and regulation in transgenic plants (Twyman, Christou & Stöger, 2002).

The adoption of DNA recombinant technology has been considered the fastest growing trend in the history of agriculture, and, over recent years, the full potential of this modern biotechnology has been exploited for its application in modern plant breeding. For centuries,

conventional plant-breeding programs have produced crops with new traits and improved quality and yields. Classical plant breeding has been based on improving plant varieties by using different strategies that include techniques such as simple plant crossing and selection, cell tissue culture techniques, mutagenesis based on irradiation, chemical mutagenesis, or the use of transposons. DNA recombinant technology is, however, in marked contrast to traditional breeding, where undefined genes are routinely transferred among breeding lines, species, and even genera. In the context of genetic engineering, the nature of the DNA intended for transfer might be controlled in a very precise manner and limited to the exact minimal DNA sequence that can confer the desired trait. Thus, recombinant DNA technology is used to create genetically modified (GM) plants, which are used to grow GM crops. The rapid progress of this technology has opened new prospects in the development of plants for the production of food, feed, fiber, forest, and other products (Petit *et al.*, 2007).

Typically, GM plants contain an expression cassette or insert that consists of a promoter that controls the expression of the transgene, the encoding region that defines the sequence of amino acids of a particular gene that confers the novel trait, and an expression terminator that functions as a stop signal to terminate the reading of the gene during protein production (Robinson, 2001). The introduction of more than one trait is often achieved by crossing individual single-gene GM lines to produce so-called stacked gene varieties.

B. Genetically modified crops.

The development of GM crops might pursue a variety of purposes that include benefits in industrial processing and for consumer as well as agronomic productivity (Namuth & Jenkins, 2005). Industrial-processing benefits include modifications in grain- or plant-chemical profiles (for example, with respect to oils, starch, fiber, protein), but also includes plants that might produce specific chemicals, natural polymers, pharmaceuticals, decontamination

agents, or fuels (http://www.isaaa.org).). On the other hand, traits to improve productivity aim at better soil-management practices that lead to higher returns and increased profit. Among the modifications, tolerance to herbicide (De Block *et al.*, 1987) and resistance to insects and disease (Hails, 2000) are predominant in current commercialized GM crops, whereas resistance to harsh environmental conditions is still under development.

Since the first commercialization of GM tomato in 1994, over one hundred GMOs have been approved by regulatory agencies in different countries (http://www.agbios.com). In the past decade, the total accumulated land areas cultivated with transgenic crops have increased dramatically. The global area of approved GM crops in 2008 was 125 million hectares in 25 countries compared with 114.3 million hectares in 2007, with an increase of 10.7 million hectares equivalent to an annual growth rate of 9.4% in 2008 (James, 2008). Today, it is possible to introduce and express DNA stably in nearly 150 different plant species (Twyman, Christou & Stöger, 2002), including many important crops such as soybean, maize, wheat, rice, cotton, potato, canola, and tobacco. Furthermore, several GM sugar beet, rice, and potato plants, which are undergoing field trials worldwide, are expected to enter the world markets in the next few years. An expected second generation of GMOs with nutritionally enhanced traits, such as, for instance, plants enriched in β-carotene (Ye *et al.*, 2000), vitamin E (Cahoon *et al.*, 2003), or omega-3 fatty acids (Kinney, 2006)] could likely enter the market in the near future (Robinson, 2001; Schubert, 2008).

C. Controversial safety issues on GMOs.

1. Environment and health concerns.

In spite of its important economic potential, recombinant-DNA technology has become highly controversial, not only within the scientific community but also in the public sector since its

beginning more than three decades ago (Berg *et al.*, 1975). Although GM plants have been farmed and marketed for over a decade, a great deal of controversy still persists about the introduction of GMOs in crops, food, and feed. As with any new technology, concerns have been raised about potential effects that might not be immediately apparent (Roller, 2001). The main controversial issues concentrate on four areas: environmental concerns [(Hails, 2000); (Wolfenbarger & Phifer, 2000); (Thomson, 2003)], concerns about potential harm to human health [(Garza, 2003); (Domingo, 2007); (Craig *et al.*, 2008)], ethical concerns interferences with nature and individual choice (Frewer *et al.*, 2004), and a combination of ethical and socio-economic concerns related to patent issues [(Vergragt & Brown, 2008); (Herring, 2008)].

2. Unintended effects in GM crops.

Regardless of the presumed accuracy of recombinant DNA technology for genetic modification, possible unintended effects that derive from the genetic transformation might occur. Unintended effects are those effects that go beyond the primary expected effects of the genetic modification, and that represent statistically significant differences in a phenotype compared with an appropriate phenotype control (Cellini *et al.*, 2004). Unintended effects might be potentially linked to secondary and pleiotropic effects of gene expression, and, in some cases, they could be somehow predicted or explained from our current knowledge of plant biology and metabolic pathway integration and interconnectivities, or from the function of a transgene or the site of genomic integration [(Kuiper *et al.*, 2001); (Ali *et al.*, 2008)]. However, some other unintended effects might be associated with different alterations that occur during the transformation and tissue-culture stages of GMO development (Latham, Wilson & Steinbrecher, 2006). In this regard, unexpected transformation-induced mutations as the result of deletions, insertions, rearrangements (including duplications), inversions, and

translocations in and outside the genome insertion-site of GM plants have been widely reported in the literature (Latham, Wilson & Steinbrecher, 2006). For instance, genome rearrangements and the presence of foreign-DNA sequences have been detected in commercially approved GM cultivars that were selected for single insertion events [(Fitch *et al.*, 1992); (Windels *et al.*, 2001); (Hernandez *et al.*, 2003); (Rosati *et al.*, 2008)]. These types of mutations produce loss, acquisition, and altered or aberrant expression of important traits, and consequently, could affect the phenotype of the GM plant. The unintended effects derived from this type of genomic alterations are unpredictable and difficult to explain without the thorough characterization of the plant at the molecular level. In other cases, unintended genetic effects will only be observed if they result in a distinct phenotype, including compositional alterations that could be undetectable with the analytical approaches used in conventional risk assessments. From the safety perspective, these unintended effects represent a significant source of unpredictability that might have an impact on human health and, the environment (Ioset *et al.*, 2007).

D. Legislation and safety assessment of GM crops.

Because of the complex composition of foods, safety assessment for GM-crop-derived foods is not a straightforward task as discussed in the literature (Kuiper & Kleter, 2003). As a consequence, important efforts have been made in order to establish globally agreed guidelines for the safety assessment of food and food ingredients derived from GM crops. A general leading strategy has been based on the assumption that traditional crop-plant varieties currently on the market that have been consumed for decades have gained a history of safe use (Kok & Kuiper, 2003), and, therefore, they can be used as comparators for the safety assessment of new GM crop varieties derived from established plant lines. This concept, referred to as “substantial equivalence” (OECD, 1993) or “comparative safety assessment”,

constitutes the basis for the current safety assessment of GM foods in many countries. Application of this concept requires the comparison of the GM crop and an appropriate 'safe' comparator according to the agronomical and morphological characteristics, and the chemical composition, including macro- and micro-nutrients, key toxins, and key anti-nutrients (König *et al.*, 2004). However, the application of the substantial "equivalence concept" cannot be considered as a safety assessment *per se*, but rather enables the identification of potential differences between the existing food and the GM crop-derived food; those differences should be further investigated with respect to their toxicological impact (Kuiper *et al.*, 2002).

E. Analytical strategies for the study of GM-crop composition.

In general terms, two conceptually and methodologically different analytical approaches have been used to study GM crops: targeted analysis and profiling approaches. Targeted analysis is helpful to study of the primary or intended effect of the genetic modification. In some cases, the interest might be focused on the insertion and the expression of the new transgene; subsequently, the analysis is directed towards the detection of specific DNA, mRNA, or proteins (i.e., target analytes). Also, with the goal to study the intended effect of the genetic modification at the metabolite level, target analysis might also focus on the detection of a limited selection of metabolites that are involved in altered biochemical/physiological pathway in the GMO. Moreover, the application of targeted analysis to characterize a number of constituents, including macro- and micronutrients, antinutrients, and natural toxins in food crops, has also been proposed as a tool for comparative safety assessments of a GM crop with its traditional counterpart [(Cellini *et al.*, 2004); (Shepherd *et al.*, 2006)].

In the context of substantial equivalence, targeted analysis should cover a number of key nutrients such as proteins, carbohydrates, fats, vitamins, and other

nutritional/antinutritional compounds, which whether or not unintentionally modified, might affect the nutritional value and safety of the crop. In this regard, selection of the target compounds to be analyzed must take into consideration the species, structure, and function of the transgene or transgenes, as well as possible interferences in metabolic pathways. Nevertheless, numerous concerns have been raised about the use of such a targeted analytical approach to compare the composition of GM crops to their traditional counterparts. It has been pointed out that this approach is biased (Millstone, Brunner & Mayer, 1999), and presents many limitations, such as the possible occurrence of unknown toxicants and anti-nutrients, particularly in food-plant species with no history of (safe) use (Kuiper *et al.*, 2001). Moreover, although a few studies have identified unintended effects with targeted approaches [(Hashimoto *et al.*, 1999); (Shewmaker *et al.*, 1999); (Ye *et al.*, 2000)]; this strategy might restrict the possibilities to detect other unpredictable unintended effects that could result directly or indirectly from the genetic modification.

The aforementioned issues corroborate the need for new and more powerful analytical approaches to study the complexity of this problem, and to increase the chances to detect unintended effects. As an alternative approach, the European Food Safety Agency (EFSA) has recommended the development and use of profiling technologies such as genomics, transcriptomics, proteomics, and metabolomics, with the potential to extend the breadth of comparative analyses (EFSA, 2006). Profiling analysis at the gene, transcript, protein, and metabolite levels are methods of choice to investigate the physiology of GM plants as comprehensively as possible in order to increase consequently the chances to detect unintended effects. Furthermore, the development of more powerful analytical tools is highly required in order to address the forecoming second generation of GMOs, in which significant changes in metabolites such as polyphenols, vitamins, fatty acids, or amino acids will be introduced (Cellini *et al.*, 2004). In addition, the development and application of these

technologies for plant-composition comparison will help to study the performance and value of novel GM plants.

This review highlights the main MS-based analytical methodologies and strategies for the study of GM crops. It is mainly focused on published experimental approaches that are potentially useful to detect and understand intended, as well as unintended effects that result from the genetic transformation. Some prospects for the future are also discussed.

II. MS-BASED "OMICS" STRATEGIES FOR GM CROP ANALYSIS.

Biological systems are highly complex, regulated networks. Regulatory connections exist among all "levels" of the biological system (DNA, RNA, protein, and metabolite), and these circuits can be modulated by internal and external signals (García-Cañas *et al.*, 2009). Accordingly, it is now clear that a study of changes only in a limited group of target compounds associated with the genetic modification does not necessarily lead to an overall functional understanding. Likewise, as it has been discussed above, target-based analysis presents some limitations when applied to the investigation of possible unintended effects that result from the genetic transformation.

Recent advances in the development of high-throughput analytical techniques to investigate the composition and functions of genome, proteome, and metabolome have led to a rapid proliferation of the so-called '-omics' techniques. The development of genomics, transcriptomics, proteomics, and metabolomics has created extraordinary opportunities to increase our understanding on how a particular genetic-transformation event affects gene- and protein-expression, and ultimately influences cellular and plant metabolism.

Several laboratories have explored profiling methods with different aims, including the investigation of the composition and performance of a GM crop, as well as the detection of unintended effects [(Baudo *et al.*, 2006); (Batista *et al.*, 2008); (Coll *et al.*, 2008)]. Among these novel technologies, the gene-expression microarray is a leading analytical technology in several research fields; for instance, in plant biology (Galbraith, 2006), pharmacogenomics (Chicurel & Dalma-Weiszhausz, 2002), nutrigenomics (García-Cañas *et al.*, 2009) and the recently defined field of foodomics (Cifuentes, 2009). A gene-expression microarray is based on specific nucleic acid hybridization, and can be used to measure simultaneously the relative quantities of specific mRNAs in two or more samples for thousands of genes. In this regard, MS-based analytical methodologies are indispensable analytical tools in plant proteome and metabolome studies [(Newton *et al.*, 2004); (Villas-Boas *et al.*, 2005); (Jorrín, Maldonado & Castillejo, 2007); (Baginsky, 2009)] as will be discussed below. Moreover, when comparing a GM crop to its non-GM isogenic variety, it is important to grow both varieties under identical conditions to avoid the influence of other variability factors such as soil, water, weather, etc.

In protein- and metabolite-profiling, the extraction of analytes is a major aspect to take into account. The choice of a particular extraction method will be in accordance with the goal of the study. In this sense, it is important to bear in mind that all extraction techniques constitute a compromise. Unlike target analysis, where the extraction parameters for certain target compounds can be optimized, profiling analysis will cover a range that is as broad as possible of extracted compounds, at the price of potentially low extraction efficiency for some analytes. In opposition to genomics and transcriptomics, where the analytes share the same physicochemical properties, the major limitations in proteomic and metabolomic profiling are associated with the heterogeneity of analytes in terms of physicochemical properties and the extreme differences in abundance. For example, a proteome can have a dynamic range of 7-12 orders of magnitude, and only a few orders can be analyzed simultaneously with the current

proteomic platforms. Owing to this complexity, it is important to develop suitable methodologies of extraction, prefractionation and/or enrichment of less-abundant proteins, and separation procedures in order to simplify the mixtures and to allow their efficient separation and identification with MS-based analytical methodologies [(Righetti *et al.*, 2005); (Haynes & Roberts, 2007)]. 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) by MS-based analysis.

A. MS-based approaches for proteomic profiling.

The genetic modification might entail variations in a number of proteins, many of whose functions might not be known; these variations make challenging the study of the biological significance of such changes. In order to glean an insight into how the modification of the genetic content produces alterations in the plant proteins, a comparative proteomics strategy is mainly used. A combination of three technologies is mostly employed for this goal: two-dimensional gel electrophoresis (2-DGE) to separate complex protein mixtures, image analysis to compare 2-DGE gels, and MS to determine the identity of the differentially expressed proteins. 2-DGE is the most commonly used analytical methodology to monitor changes in the expression of complex protein mixtures, and provide the highest protein-resolution capacity with a low-instrumentation cost. However, this methodology has some limitations. Thus, in addition to the 2-DGE technical hitches to separate highly hydrophobic, extreme isoelectric point or molecular weight (MW) proteins, one of the major sources of error in 2DE is the gel-to-gel variation that makes difficult an exact match of spots in the image-analysis process. Differential in-gel electrophoresis (DIGE) avoids some of the reproducibility problems by loading different samples labeled with ultrahigh-sensitive

fluorescent dyes in the same gel (Timms & Cramer, 2008). After image analysis of the 2-DGE (or DIGE) gels, protein spots of interest are submitted to an in-gel digestion step with an endoproteinase of known specifity. Currently, in the so-called bottom-up proteomic approach (see Figure 1), MS (typically MALDI-TOF MS) and different variants of LC-MS are the established methodologies to analyze a peptide mixture from a 2-DGE separated protein digested with a certain protease enzyme. Databases of protein sequences are used in different ways for protein identification. The main limitation of the *bottom-up* approach is that information obtained is related to a fraction of the protein; information about posttranslational modifications (PTM) might be lost if the PTM-bearing peptide/amino acid is not detected..

Representative examples of the application of MS-based proteomic analysis to the study of substantial equivalence of GM crops are listed in Table 1. As can be seen in Table 1, most of the published proteomic works used the 2-DGE (or DIGE) technology, followed by the identification of the species with a MS-based *bottom-up* proteomic approach. Some representative examples are next discussed.

Thus, a comparison of protein profiles of a GM tomato with a genetically added resistance to tomato spotted-wilt virus (TSWV) vs. the same unmodified tomato line was carried out; no significant differences were detected, either qualitative or quantitative (Corpillo *et al.*, 2004). In another study, the expression of recombinant antibodies in two transgenic crops (tomato and tobacco) -as a strategy to confer self-protection against virus attack- did not significantly alter the leaf-proteome profile (Di Carli *et al.*, 2009). However, Rocco *et al*. observed that a tobacco transformed with the tomato prosystemin gene affected the expression of a number of proteins involved in protection from pathogens and oxidative stress and in carbon/energy metabolism (Rocco *et al*., 2008). When GM maize was studied, unexpected differences between Bt and wild-type maize were observed [(Albo *et al.*, 2007); (Zolla *et al.*, 2008)]. The great complexity of the proteomic-data interpretation was stated by

Zolla *et al.*, because a significant number of proteins were differentially regulated by environmental influence and as a result of the gene insertion; the environment affects protein expression more than gene manipulation (Zolla *et al.*, 2008). GM wheat cereal has also been studied from a proteomic point of view. Two GM durum wheat cultivars (namely, Svevo B730 1-1 and Ofanto B688 1-2) with modified functional performance of the grain were investigated (Di Luccia *et al.*, 2005). When prolamin composition of both manipulated lines was compared to their respective control lines, significant differences were found only in the GM Ofanto wheat cultivars (Di Luccia *et al.*, 2005). In a later study, it was observed that several classes of proteins were differentially accumulated in the subproteome endosperm of wheat as a result of the overexpression of a low molecular weight glutenin subunit (LMW-GS) in a GM bread wheat that had modified visco-elastic properties of the derived dough (Scossa *et al.*, 2008). The overexpression of LMW-GS was compensated by a decrease in the amount of polypeptides that belong to the prolamin superfamily. According to the authors, most of the observed variations included predictable alterations of the seed proteome. Lehesranta *et al.* carried out a study on proteome diversity on a large selection of potato varieties and landraces, and showed significant quantitative and qualitative differences in most of the detected proteins (Lehesranta *et al.*, 2005). However, when different GM potato lines were compared with their controls, statistical analysis showed no clear differences in the protein patterns. In a different study, it was observed that the MALDI-TOF MS profile of low molecular mass proteins of non-GM and GM potato (in which the expression of the G1-1 gene was inhibited with antisense technology) did not show any significant differences when the complete tuber was studied (Careri *et al.*, 2003). On the contrary, several differences were observed in the *m/z* range 3447-6700 when the proteome of apical eyes of the same potato tubers was studied; those data demonstrated that the G1-1 gene is mainly expressed in this tissue.

In general, there is limited information on the extent of natural variation in the proteome of plants caused by environmental factors. In order to avoid any mis- or over-interpretation of the results and any misplaced on safety concern, the variability of the protein expression in conventional crops grown under a range of different environmental conditions should be studied first. Following this idea, the model plant *Arabidopsis thaliana* was used by Ruebelt *et al.* to carry out a broad study, in which analytical validation of 2-DGE methodology was initially done (Ruebelt *et al*., 2006a) and applied to better understand the natural variability of the proteome (Ruebelt *et al*., 2006b). The final goal would be to carry out a comprehensive proteomic study to detect unintended effects (Ruebelt *et al*., 2006b). The seed proteomes from twelve *A. thaliana* lines were compared to those of their parental lines in the context of natural variability; it was observed that the genetic modification from three different genes and three different promoters did not cause any unintended changes (Ruebelt *et al*., 2006c).

Gel-free protein (or peptide) separation methods enable the direct coupling of a chromatographic or electrophoretic analytical separation technique to a mass spectrometer, so that the separated species that elute from the column can be *on-line* detected and characterized with MS or MS/MS. Gel-free protein separation methods have higher capabilities to analyze highly hydrophobic and extreme isoelectric point or MW proteins. Moreover, the main advantages of a gel-free protein separation method coupled to MS compared with conventional 2-DGE and subsequent MS-based analysis of the protein (or digested protein) of interest are: i) the possibility of full automation; ii) the lower amount of needed starting material; iii) the potential high-throughput capabilities; and iv) the better reproducibility in terms of qualitative (analysis time) and quantitative (peak area) analysis.

Liquid chromatography (LC) and capillary electrophoresis (CE) in their different modes are the main gel-free methodologies applicable to the separation of complex protein (and peptide) mixtures due to their high resolving power and their potential for full automation and high sampling rates [(Tang *et al*., 2008); (Chen *et al*., 2008); (Herrero, Ibañez & Cifuentes, 2008); (Sandra *et al*., 2009)]. Moreover, in 2-DGE, relative abundances of proteins in the samples under study are compared before MS analysis, whereas in LC- or CE-MS the comparison of peptides (or proteins) is carried out after the MS data have been acquired. Multidimensional coupling of CE and LC, and subsequent on-line detection by MS, is a promising methodology in proteomic applications as an alternative to 2-DGE to separate of complex peptide (and in less extent, protein) mixtures (Shen & Smith, 2002). Recently, CE-ESI-MS was applied for the analysis of the zein-proteins fraction from different maize cultivars. Two different mass analyzers were studied; i.e., TOF, and IT (Erny *et al.*, 2008). Although both instruments provided good results in terms of sensitivity and repeatability, CE-ESI-TOF MS identified a higher number of proteins. The CE-ESI-TOF MS coupling was applied to the study the zein fraction of three GM maize lines (Aristis Bt, Tietar Bt and PR33P66 Bt) and their corresponding control lines; no significant differences were found between the GM line and its wild counterpart (Erny *et al.*, 2008).

Profiling proteomic approaches that use MS-based methodologies are also essential to study the mechanisms involved in the response of GM plants submitted to a variety of biotic (pathogens, parasites, etc.) and abiotic (chemicals, drought, salinity, etc.) stresses. Thus, in spite of the aforementioned advantages of a gel-free protein separation method coupled to MS, a differential expression proteomics strategy that combines 2-DGE and MS is still the dominant analytical platform to investigate how the genetic modification produce alterations in proteins abundance, structure, or function, as well as to study the relationships between stress-induced proteins and up- or down-regulated proteins. In this sense, 2-DGE followed by

MALDI-TOF MS was used to study the effect of drought stress on the proteomic expression of a herbicide-resistant transgenic wheat; drought affected the expression of low MW proteins, in the range of 15-27 kDa with isoelectric points between 6.5 and 7.5 (Horvath-Szanics *et al.*, 2006). A similar expression proteomics study was performed to assess changes in the transgenic leaf proteome from leaves of GM tobacco with perturbed polyamine metabolism caused by an S-adenosylmethionine decarboxylase (AdoMetDC) overexpression (Franceschetti *et al*., 2004). Identification of the proteins with MALDI-QTOF MS showed that the isoforms of chloroplast ribonucleoproteins decreased in abundance in the three transgenic tobacco lines that overexpressed the AdoMetDC protein. A differential protein expression approach was also used to study the effect of variation in alcohol dehydrogenase expression (ADH) in GM grapevine leaves (Tesniere *et al.*, 2006). After observing (in a previous study) variations in some aspects of primary and secondary metabolism, significant alterations were found at the proteomic level (Sauvage *et al*., 2007). From the 14 selected up- or down-regulated spots from the 2-DGE gels, 10 proteins (from 9 spots) were identified with MALDI-TOF MS, and 9 proteins (from 5 spots) were identified with LC-QTOF MS; most identified proteins were related to chloroplasts or to primary metabolism. The effect of the overexpression of calcium-dependent protein kinase 13 (CDPK13) and calreticulin-interacting protein 1 (CRTintP1) involved in cold-stress response was studied in GM rice with non-targeted proteomic approaches (Komatsu *et al*., 2007). Six 2-DGE-separated proteins related to cold signaling were identified with ESI-QTOF MS.

One of the main factors that make difficult the analysis of complex proteomes is the presence of abundant species. The importance of low-abundance protein enrichment in a GM model plant was showed by Widjaja *et al.* (Widjaja *et al*., 2009). Patterns of two isogenic *Arabidopsis* lines with the same dexamethasone-inducible *avrRpm* transgene that differ in the absence or presence of the *RPM1* gene were compared by a study of the microsomal

subproteome. In a different study, an enhanced resolution and protein coverage was obtained with microsomal fractionation and rubisco (with up to 50% of the soluble protein in leaves) depletion with differential concentration of the polyethylene glycol (PEG)-mediated protein-fractionation technique (Kim *et al*., 2001). A total of 34 differentially regulated protein spots could be identified; most were metabolism-, signaling-, and defense-related proteins.

Shotgun-proteomic approaches have also been used in non-targeted studies of GM crops. In shotgun-proteomics, protein digestion is performed without any prefractionation/separation of the proteome, and peptides are separated with LC followed by MS/MS analysis to provide a comprehensive, rapid, and automatic identification of proteins in complex mixtures. Currently, this approach seems to be the best choice to analyze samples that cannot be efficiently resolved on 2-DGE because of their physico-chemical properties. Shotgun-proteomics with 4-plex iTRAQ (isobaric tags for relative and absolute quantification) reagents identified and quantified a rice proteome for comparative expression profiles between transgenic and wild-type (Luo *et al*., 2009). The four independent isobaric reagents (designed to react with all primary amines of a protein hydrolyzate) reacted with four different protein hydrolyzates that were subsequently pooled. MS/MS analysis of four unique reported ions *(m/z*=114-117) were used to quantify the four different samples (Ross *et al*., 2004). Previously, it was observed that the iTRAQ *shotgun* strategy provided a more consistent protein quantitation compared to 2-DGE (Aggarwal, Choe & Lee, 2006). Among the 1883 proteins identified in rice endosperm with this analytical strategy, 103 displayed significant changes between GM and wild-type rice (Luo *et al*., 2009). Today, eight different iTRAQ are available to enable larger scale screenings with up to eight different samples in the same MS analysis.

B. MS-based strategies for metabolomic profiling.

Metabolomic studies of GM plants might indicate whether intended and/or unintended effects have taken place as a result of genetic modification (Shepherd *et al*., 2006). However, as in the case of proteomics, at the moment there is no simple analytical platform to acquire significant amounts of data in a single experimental analysis to provide a maximum coverage of the metabolome. Metabolites encompass a wide range of chemical species that have widely divergent physicochemical properties. In addition, the relative concentration of metabolites in a cell or tissue can range from the millimolar to the picomolar level. Consequently, high resolution and sensitivity are the most relevant parameters to take into account to select an appropriate method for comprehensive metabolomic analysis (Villas-Boas *et al*., 2005).

In essence, metabolic profiling approaches can be divided into nuclear magnetic resonance (NMR) and MS-based methodologies. NMR approaches are out of scope of this review; therefore, we will only discuss MS-based procedures. The main advantages of MS are high resolution, high sensitivity, a wide dynamic range, coverage of a wide chemical diversity, robustness, and feasibility to elucidate the MW and structure of unknown compounds. MS is inherently more sensitive than NMR, but it is destructive and it is generally necessary to employ different extraction procedures and separation techniques for different classes of compounds (Lu *et al*., 2008).

MS has wide possibilities to evaluate GM crops based on their metabolic profiling, as demonstrated through the large number of applications that use GC-MS, LC-MS, CE-MS, or MS as a stand-alone technique (Hoekenga *et al*., 2008). A summary of some of these MS-based profiling approaches for the analysis of GM crops is given in Table 2; these applications are discussed next, and are classified according to the analytical tool employed.

1. Gas chromatography-mass spectrometry (GC-MS).

GC coupled to MS has been extensively used for metabolome analysis because of its high separation efficiency, reproducibility, and the ease to interface GC with different MS analyzers (Villas-Boas *et al*., 2005). GC-MS can be used to analyze a wide range of volatile compounds, and semi- and non-volatile compounds after chemical derivatization. Recently, GC-MS has been established as one of the most versatile and sensitive techniques for metabolic profiling. The use of this technique combined with a variety of chemometric approaches (e.g., principal components analysis, PCA) has been proven to be suitable to discover differences that enable plants of distinct lines to be distinguished from each other (Fiehn *et al*., 2000).

In a series of papers, Roessner *et al*. applied this profiling methodology to identify and quantify the level of the main metabolites in tubers of transgenic potato lines with altered sugar or starch metabolism (Roessner *et al*., 2000). The first study reported a methodology based on the extraction of polar metabolites from potato tubers, followed by methoximation and silylation to volatilize various classes of compounds (Roessner *et al*., 2000). After sample treatment, the analysis resulted in complex and reproducible GC-MS chromatograms of more than 150 compounds; 77 compounds of known structure were identified by comparison of the obtained spectra with commercially available spectra from MS libraries. GC-MS corroborated the scientific conclusions drawn in previous studies of the GM lines; in particular, the increase of glycolysis, amino acids, and organic acids observed in the GMOs. In addition, unexpected alterations of the levels of some disaccharides such as trehalose were identified. In further reports, GC-MS analysis of potato tubers that were genetically modified to contain more efficient sucrose catabolism revealed a massive elevation in the content of each individual amino acid [(Roessner, Willmitzer & Fernie, 2001a); (Roessner *et al.*, 2001b)]. This unexpected feature was particularly surprising because the tuber did not possess the necessary

machinery for *de novo* synthesis of amino acids; those results suggested that pathways other than those targeted by the genetic modification could be affected. Additionally, the method was evaluated in combination with data-mining tools that included hierarchical clustering and PCA to discriminate plants that were genetically modified or cultivated under different growth conditions. In a subsequent paper, the reported methodology was adapted to investigate the influence of hexokinase, a key enzyme in sucrose metabolism, in developing transgenic tomato plants that overexpressed Arabidopsis hexokinase, with a particular focus on distinct phases of fruit development (Roessner-Tunali *et al*., 2003). As an example, the GC-MS electropherogram can be seen in Figure 2. Although many interesting results emerged from a point-by-point analysis, and from a study of the changes of specific metabolites over developmental time, PCA revealed that separation of the GM fruits from the controls is larger in the early developmental stage; those data suggested a higher influence of the recombinant enzyme on the metabolism at this stage.

After these pioneering works, a number of GC-MS studies have been reported. For instance, by following a similar approach, metabolic profiling of a tryptophan (Trp)-enriched GM soybean line has recently shown significantly higher levels of fructose, myo-inositol, and shikimic acid among 37 total organic acids, sugars, alcohols, and phenolic compounds in the leaves compared to the controls (Inaba *et al*., 2007). Likewise, GC-MS metabolic-profiling analysis of a transgenic variety of *Artemisia annua* L., the natural source of the anti-malarial drug artemisinin, has recently proven to be a valuable tool to identify key enzymes in the biosynthesis pathway of this phytochemical (Ma *et al*., 2008). The genetic modification involved the overexpression of farnexyl diphosphate synthase, an important enzyme in sesquiterpenoid biosynthesis. Extracts from different plant tissues were derivatized for further GC-MS analysis to provide, after chromatogram alignment, 188 chromatographic peaks that were evaluated with PCA or PLS-DA (partial least squares discriminant analysis). The

experiments demonstrated different sesquiterpene contents in different developmental stages and strains; those data suggested the existence of a potential key step in the biosynthetic pathway of artemisinin (Ma *et al*., 2008).

Plants have the ability to produce volatile aroma compounds, such as aldehydes and alcohols, which give rise to characteristic flavors and odors. Flavors are influenced by numerous factors, mainly, genetic makeup and external agronomical factors, such as climate and soil type. Volatile aromatic compounds are secondary metabolites that are generated through numerous pathways during the fruit-ripening process. Malowicki *et al*. have carried out a comparative GC-MS study of the volatile composition of virus-resistant transgenic and conventional raspberry grown in two different locations and seasons (Malowicki, Martin & Qian, 2008). Volatile compounds were extracted from the red raspberries with a stir-sorptive bar. MS quantification was carried out with selective-mass ions to avoid any interference between coeluted compounds. Quantification curves were constructed by plotting the selective-ion abundance ratio of target compounds with their respective internal standards against the concentration ratio. None of the 30 selected compounds, based on their previously reported importance to raspberry aroma as well as their representation to various chemical classes including alcohol, aldehyde, ketone, ester, terpene, and terpene alcohol, showed any difference between the transgenic lines and the wild type. In addition to raspberry, the aroma from transgenic cucumber lines have also been evaluated with GC-MS (Zawirska-Wojtasiak *et al*., 2009). Four lines of GM cucumber with different levels of thaumatin II gene overexpression were tested. Two extraction methods of volatile compounds from cucumbers were evaluated; namely, microdistillation (MD) and solid-phase microextraction (SPME). The cucumber extracts were subjected to GC-EI-Q MS and GC-EI-TOF MS analysis. SPME enabled the identification of a higher number of compounds (a total of 28 compounds) due to its capability to detect low boiling point volatiles, defined by the solvent in the case of MD.

Although all identified compounds were identical in the transgenic lines and in the control, analyses showed that, regardless the MS-based analytical technique used, significant differences occurred in the quantitative composition of the aroma between fruits of transformed and control cucumber lines.

Among the novel developments in metabolomic profiling of GMOs, new chemometric approaches include modeling of two or more classes, such as the developed based on orthogonal PLC discriminant analysis (OPLC-DA) to compare two different transgenic poplar lines (*Populus tremula* L.) with the wild-type with GC-MS metabolomics (Wiklund *et al*., 2008). The two transgenic lines were up- and down-regulated for the expression of *PttPME1* gene, respectively, with the aim to affect the degree of methyl esterification of homogalacturonan, the most important component of pectin in plant cell walls. Poplar metabolites were extracted with organic extraction from leaves, and were derivatized for GC-EI-TOF MS separation. Data sets were processed with the hierarchical multivariate curve resolution MATLAB script, which is useful for spectra comparison in the National Institute of Standards and Technology (NIST) library. In addition, an improved visualization and discrimination of interesting metabolites from wild and GM lines could be demonstrated with OPLS (Wiklund *et al*., 2008).

Recently, several laboratories directed their studies toward the investigation of unintended effects in GM crops. Bernal *et al*. developed a methodology based on supercritical fluid extraction (SFE) and GC-EI-Q MS for the selective extraction and subsequent profiling and quantification of amino acid from GM soybean and maize (Bernal *et al.*, 2008). The suitability of the method to identify differences in amino acids profiles were confirmed by the comparison of five different transgenic lines with their corresponding isogenic lines grown under the same conditions. Catchpole *et al*. proposed a hierarchical experimental approach to study the compositional similarities/differences between GM and conventional crops

(Catchpole *et al*., 2005). The methodology was tested in potato crops that were genetically modified to contain high levels of inulin-type fructans. The approach involved an initial evaluation of the degree of compositional similarity between tubers of transgenic and several conventional potato cultivars. This first step was carried out with FIA-TOF MS (flow-injection analysis ESI-MS) of 600 potato extracts and subsequent PCA that identified 15 top-ranking ions for genotype separation with higher-loading scores; some ions corresponded to oligofructans of different polymerization degrees. Complementary GC-EI-TOF MS profiling of more than 2000 tuber samples provided a more-detailed global profiling of 242 individual metabolites (90 positively identified, 89 assigned to a specific metabolite class, and 73 unknowns). Further chemometric analysis of data showed that, apart from targeted changes by the genetic modification, transgenic potatoes displayed a similar metabolite composition inside the range exhibited normally by conventional cultivars. In a recent paper, Zhou *et al*. reported the combined use of GC-flame ionization detection (FID) and GC-MS to investigate possible unintended effects in a transgenic line of rice that expressed two genes that confer distinct insect resistance (Zhou *et al*., 2009). GC-MS was exclusively used to identify certain important compounds after GC-FID profiling. Authors employed multivariate analyses, namely, PCA and PLS-DA, to visualize and analyze the metabolite data. They concluded that the growing conditions and the genetic transformation induced a similar influence on the concentrations of glycerol-3-phosphate, citric acid, oleic acid, and sucrose, whereas other metabolites (sucrose, mannitol, and glutamic acid) were widely affected by the genetic modification.

2. Liquid chromatography-mass spectrometry (LC-MS).

In addition to GC-MS, LC coupled to MS is a useful tool for the metabolomic analysis of GM crops, and provide a wide dynamic range, reproducible quantitative analysis, and the ability to separate and analyze extremely complex samples (Lu *et al*., 2008). Moreover, LC-MS is

considered a versatile technique for the analysis of metabolites, whose analysis with GC-MS is, in general, precluded, These metabolites include polar/non-volatile, large, and/or thermolabile compounds. In addition, LC-MS can resolve and quantify multiple components in crude biological extracts typically down to the nanomolar or picomolar range from as little as microliter volumes.

The application of LC-MS to metabolite profiling of GM crops is relatively recent. In general, metabolite-profiling studies of GMOs with LC–MS have been mostly performed with solvent gradients and reversed-phase LC (RPLC). LC-MS is useful to provide complementary and interesting data in the investigation of metabolism alterations in transgenic grapevine (*Vitis vinifera*) (Tesniere *et al*., 2006). Grapevine plants transformed with three different genetic constructions (normal, sense, and antisense) to either over- or underexpress alcohol dehydrogenase were characterized with different molecular methods, biochemical and profiling techniques. More precisely, profiling of phenolic compounds was performed with LC-ESI-IT MS, whereas volatile compounds were profiled with GC-EI-MS. Among the profiles from transgenic grapevine with normal, sense, and antisense constructs, differences were noted in some phenolic compounds and volatile secondary metabolites that belong to the classes of monoterpenes, C12-norisoprenoids, and shikimates (Tesniere *et al*., 2006). As exemplified in this study, the combination of different analytical techniques allows a better description of the metabolome status of a GMO.

Recently, the LC-MS profiling of polyphenols in GM crops has attracted the attention of several laboratories. Shin *et al*. used this technique to explore the flavonoids content in GM rice endosperm that expresses regulatory genes from maize that induce the production of various flavonoids (Shin *et al*., 2006). Ioset *et al*. have recently investigated changes in the metabolite accumulation in two transgenic lines of wheat (*Triticum aestivum* L.) with either antifungal or viral resistance (Ioset *et al*., 2007). Flavonoids were extracted with SPE, and

were analyzed by LC-IT MS with two different ionization sources, ESI and APCI. In addition, LC-MS/MS experiments, using the ESI in negative mode, were performed after selection and consecutive fragmentation of the most intense precursor ions. Based on their MS/MS fragmentation, this analytical procedure allowed a differentiation between *C*-glycoside flavonoids and *O*-glycoside analogues; that differentiation was especially advantageous to draw structural conclusions about the flavonoids. Hierarchical clustering of data revealed a closer correlation between GM/non-GM plants of the same variety than between conventional plants of different varieties. In a different study, Nicoletti *et al*. concentrated on the LC-MS profiling of stilbenes, a specific class of polyphenols, in transgenic tomato (Nicoletti *et al*., 2007). The GM tomato overexpressed a grapevine gene that encoded the enzyme stilbene synthase. The plant was designed to synthesize new compounds (trans-resveratrol and trans-piceid), and to increase total antioxidant activity. Consequently, the study was conducted to investigate possible perturbations on the synthesis of other metabolites along the flavonoids pathway. Flavonoid extracts from tomato fruits and peels were analyzed with LC-ESI-MS in the negative ionization mode, which resulted in higher sensitivity and lower background noise than in the positive mode for the detection of stilbenes and phenolic compounds. On the basis of the retention times and UV and MS data, the identification of resveratrol and its glycosilated forms was possible in one analysis. Results indicated differences in the levels of rutin, naringenin, and chlorogenic acid found in transgenic tomatoes in comparison to the control lines; those differences seem to be related to the genetic transformation (Nicoletti *et al*., 2007).

3. Capillary electrophoresis-mass spectrometry (CE-MS).

CE-MS can be considered as a complementary analytical technique to LC-MS and GC-MS. It is better suited to analyze ionic and polar thermolabile compounds that might not be separated with the reversed phase columns that are mostly used in LC-MS nor analyzed by GC-MS due

to the required high temperatures. The main advantages of CE-MS are fast separation speed and extremely high efficiency and resolution. Moreover, samples analyzed by CE-MS usually require little pretreatment. On the other hand, the sample volumes are very low and confer moderate sensitivity to CE-MS. Besides, the different ESI interfaces developed for CE-MS still have to improve their robustness.

CE-MS has already shown its potential to analyze complex metabolomes [(Babu *et al*., 2006); (Monton & Soga, 2007); (Song *et al*., 2008); (Ramautar, Somseng & de Jong, 2009)]. Thus, around 1700 different metabolites were detected (of which 150 were identified) with CE-MS from bacteria-cell extracts with two different methods and scanning from *m/z* 70 to 1027 in intervals of 30 Th (Soga *et al*., 2003). Moreover, in a recent paper, single cells and subcellular metabolomes could also be investigated with CE-MS (Lapainis, Rubakhin & Sweedler, 2009). Some attempts have been made to carry out metabolome analysis in higher plants with CE-MS methods [(Sato *et al*., 2004); (Edwards *et al*., 2006); (Harada *et al*., 2008)]. CE-MS has also been used for the non-targeted analysis of some GM crops; namely, rice, soybean, and maize. The metabolome of GM rice that overexpress YK1, which possesses dihydroflavonol-4-reductase activity and shows biotic and abiotic stress tolerance (enhancement of tolerance to ultraviolet irradiation, salt, submergence, hydrogen peroxide, and blast disease), was studied (Takahashi *et al*., 2006). MS analysis was carried out in the positive ionization mode to detect amino acids, and in the negative ionization mode to analyze organic acids, to quantitatively compare their levels in transgenic rice that express the YK1. Analytes were identified by comparison of their *m/z* values and migration times with standard metabolites. Although this study did not show significant differences in the total amount of free amino acids, a slight decrease in aspartate and glutamine were observed, most probably due to the activation of the NAD synthetic pathway induced by the overexpression of YK1, because these amino acids are precursors of NAD in plants (Takahashi *et al*., 2006). In a

different work, a chiral CE-ESI-TOF MS method was developed to study differences in the amino acid profile among six varieties of conventional and GM soybean with resistance to the herbicide glyphosate (Giuffrida *et al*., 2009). Novel modified cyclodextrins (mCDs) were used as chiral selectors in the separation buffer to obtain a good chiral resolution. The mCD concentration was so low (0.5 mM mCDs) that a direct entrance to the ESI-MS was possible with only a very low sensitivity decrease. Evaluation of D/L-amino acids from transgenic and conventional maize was carried out with this new chiral CE-ESI-TOF MS method; a very similar D/L-amino acid profile was obtained for wild and transgenic soya. However, an interesting finding was the presence of a very low amount of D-Arg in transgenic maize and not in the conventional one; however, it was concluded that a higher number of analyses should be carried out in order to discard D-Arg appearance in GM maize due, e.g., to environmental variations or natural variability. Other studies with CE-MS for metabolite profiling of GM crops used a complete analytical method of extraction, analysis, and data evaluation for transgenic and conventional maize and soybean [(Levandi *et al*., 2008); (Garcia-Villalba *et al*., 2008)]. CE-ESI-TOF MS was used to evaluate statistically significant differences in the metabolic profile of varieties of conventional and transgenic Bt11 maize (Levandi *et al*., 2008). The extraction procedure with ultrasound and different solvents was optimized in order to extract the highest number of metabolites from the maize flour. ESI-TOF MS was used to take advantage of its great mass accuracy for metabolite identification. After introducing a molecular formula into different databases, such as KEGG (Kyoto Encyclopedia Gene and Genome) or Chemspider (Database of Chemical Structures and Property Predictions), 27 different metabolites were tentatively identified, as can be seen in Figure 3. After PCA of the CE-MS set of data, some statistically significant differences between conventional and transgenic maize were found; e.g., L-carnitine and stachydrine were overexpressed in all the studied GM maize varieties (Levandi *et al*., 2008). In a similar

study, CE-ESI-TOF MS was used to compare metabolic profiles from a transgenic soybean (glyphosate-resistant) and its corresponding nontransgenic parental line (García-Villalba *et al*., 2008). In that study, 45 different metabolites, among them, isoflavones, amino acids, and carboxylic acids, were identified. The slight differences found in the metabolic profiles of both lines emphasized a clear down-expression of the three amino acids, proline, histidine, and asparagine in the GM soybean. On the other hand, a metabolite tentatively identified as 4-hydroxi-L-threonine disappeared in the transgenic soybean compared to its parental non-transgenic line (García-Villalba *et al*., 2008).

4. Fourier- transform ion-cyclotron resonance mass spectrometry (FT-ICR-MS).

The use of high magnetic-field Fourier-transform ion-cyclotron MS (FT-ICR-MS) provides the highest achievable mass resolution and accuracy to allow, in combination with soft ionization technologies, high-throughput metabolic profiling among other applications [(Marshall, Hendrikson & Jackson, 1998); (Page, Masselon & Smith, 2004); (Römpp *et al*., 2005)]. With such a high mass accuracy (sub-ppm) and ultra-high mass resolution (greater than 100,000) for component separation, elemental formula determination from hundreds of different compounds can be determined in direct infusion analyses of, e.g., crude plant extracts without a previous chromatographic or electrophoretic separation, and/or derivatization reaction. Special attention has to be paid, however, to matrix effects during direct infusion because matrix effects can produce poor ionization of interesting analytes. Moreover, FT-ICR-MS presents only moderate sensitivity and quantitative capabilities.

FT-ICR-MS-based metabolic profiling has already been used as a powerful analytical platform for plant-metabolomic studies [(Brown, Kruppa & Dasseux, 2005); (Oikawa *et al*., 2006); (Ohta, Shibata & Kanaya, 2007)]. Aharoni *et al.* published one of the first studies on the use of FT-ICR-MS to metabolomic profile GM crops (Aharoni *et al*., 2002). To obtain a

comprehensive metabolomic profile of the crude plant extract, ionization was performed in the positive and negative modes with either ESI or APCI. Interesting information was first obtained from the profiles of known metabolites during the transition from immature to ripe strawberry. The method was applied to monitor changes in the metabolic profiles of tobacco flowers that overexpress a strawberry MYB transcription factor and that are altered in petal color. From the FT-ICR-MS data set, it was observed that nine metabolites changed between transgenic and control plants, among which was the mass that corresponded to the main flower pigment, cyanidin-3-rhamnoglucoside (Aharoni *et al*., 2002). In a later study, metabolomic patterns from stress-tolerant GM rice were studied to elucidate the effects of an over-expression of the YK1 gene (Takahashi *et al*., 2005). More than 850 metabolites were determined with FT-ICR-MS in different tissues; the metabolomic profiles were significantly different among callus, leaf, and panicle. PCA also revealed slight differences in the metabolic profiles between control and YK1 in callus, which however, were almost identical those in leaf and panicle tissues (Takahashi *et al*., 2005). FT-ICR-MS was also used to examine gdhA GM tobacco (*Nicotiana tabacum*) with altered glutamate, amino acid, and carbon metabolism, which fundamentally alter plant productivity (Mungur *et al*., 2005). With the FT-ICR-MS methodology, more than 2012 reproducible ion signals could be detected; about 58% of the molecules were not in the interrogated databases, and 42% of ions were identified as known metabolites. Amino acids, organic acids, sugars, and some fatty acids significantly change their abundance in root and leaf due to the genetic modification. The altered concentration of 32 compounds with biomedical significance suggested the use of FT-ICR-MS as a useful tool for the pharmaceutical industry to discover new, interesting plant-derived compounds from GM crops. The authors recommended the use of the FT-ICR-MS data to be used as preliminary evidence to further experiments because some of the identified compounds were not plant metabolites; the measure of the exact masses could not

unequivocally identify specific compounds. CE-TOF MS and FT-ICR-MS were used to profile six varieties of maize, three GM lines with a new Cry-type gene to resist insect plagues, and their corresponding isogenic lines (León *et al.*, 2009). Pressurized liquid extraction (PLE) was used for the automated sample extraction of metabolites for subsequent FT-ICR-MS analysis. With direct infusion FT-ICR-MS in the positive and negative ESI modes, a vast amount of data was generated, from which *ca*. 1000 signals were used to assign elemental compositions. The FT-ICR-MS data were uploaded into a MassTRIX server (Suhre & Schmitt-Kopplin, 2008) in order to display the results on maize-specific annotated metabolites in the KEGG database and their related pathway maps. An example of the results from this powerful approach is shown in Figure 4. FT-ICR-MS information; however, in several cases not enough data were available to undoubtedly identify certain compounds, because FT-ICR-MS cannot differentiate among structures between isomers, so that migration time, electrophoretic mobilities, and *m/z* values provided by CE-TOF MS were used to confirm the compound identification. With this methodology, metabolic profile of the different maize lines was evaluated. Statistically significant differences were found in some metabolic pathways such as tyrosine and tryptophan metabolism. Some maize-transgenic biomarkers like L-carnitine were also observed, corroborating the previously published results (Levandi *et al*., 2008). The comparison of these two different MS-based analytical approaches showed that, although mass accuracy is very useful information for metabolite elucidation and high resolution provided many more detected metabolites, the FT-ICR-MS data must be, in some cases, complemented with additional analytical information to unequivocally identify certain compounds.

III. TARGET-BASED APPROACHES FOR GM CROP ANALYSIS

Two types of macromolecules, specific for a genetic modification, have been targeted in order to reveal the presence of GMOs (or a derivative) in foods: proteins and DNAs. Recently, a number of analytical procedures available for GMO detection in the food and feed chain involve the use of PCR because of its high sensitivity and specificity (García-Cañas, Cifuentes & González, 2004a). New trends in this field include: replacement of classical agarose gel electrophoresis with capillary electrophoresis with laser-induced fluorescence detection [(García-Cañas, González & Cifuentes, 2002a); (García-Cañas, González & Cifuentes, 2002b)]; microarray analysis for high-throughput GMO screening (Hamels *et al.*, 2009); development of biosensors (Karamollaoglu, Oktema & Mutlub, 2009); development of real-time PCR (Hernández *et al.*, 2004) and competitive PCR methods (García-Cañas, Cifuentes & González, 2004a) for GMO quantification; and development of multiplex PCR-based strategies [(García-Cañas, Cifuentes & González, 2004b); (García-Cañas & Cifuentes, 2008); (Heide *et al*., 2008)]. These developments are beyond the scope of this article, and excellent reviews on these topics can be found elsewhere [(Elenis *et al.*, 2008); (Michelini *et al*., 2008); (Marmiroli *et al*., 2008); (Morisset *et al*., 2008)]. In addition to those strategies, LC-MS has been recently demonstrated to be suitable for the multiple and simultaneous analysis of specific transgenic DNA sequences for the detection of Roundup Ready soybean, a transgenic soybean resistant to the herbicide glyphosate (Shanahan *et al*., 2007). The LC-MS approach was based on a first DNA amplification step that covered specific DNA sequences (transgenic and endogenous gene) with PCR in DNA extracts from soybean samples, followed by single base-pair extension with specific oligonucleotides and dideoxynucleotide triphosphates. Oligonucleotides generated in this step were online purified prior to LC-ESI-MS analysis. In that study, a C18 stationary phase was used because of its

ability to retain the analytes, while also allowing the use of MS-compatible buffers. However, it was necessary to minimize the buffer concentration to a 0.5 mM ammonium hydrogen carbonate and to mantain the pH below 7 in order to preserve column life. In addition to those steps, the desalting step was an essential requirement to reduce the production of adducts that decrease the sensitivity. Although the methodology reported good sensitivity and quantitative potential, it is far from being considered a routine procedure for GMO detection.

A. MS-based approaches to analyze target proteins.

Despite the current prominent role achieved by DNA detection methods in GM crops, the detection of newly expressed proteins has also been important for the investigation of the intended effect that results from a genetic modification, for example, to monitor recombinant plant-produced pharmaceutical and industrial proteins (Goldstein & Thomas, 2004), or especially, on the detection of transgene expression in the postharvest stage (Carpentier et al., 2008). Among the existing protein-based analytical approaches, the use of polyclonal antibodies for immunochemical detection has been frequently demonstrated (Grothaus *et al*., 2006). Owing to the high specificity of the immunological reaction, recognition of the target protein has been achieved (Stave, 2002). In these immunological analyses, the presence of interfering compounds must be carefully monitored because they frequently give rise to unwanted cross-reactions.

There are only few studies published on the use of MS approaches for the target analysis of transgenic protein in GM crops. Some limitations of the application of MS to protein target analysis might rely on the low expression levels of the recombinant protein in addition to the fact that, frequently, the new protein is not evenly distributed in the plant

tissues. Moreover, a significant drawback in most targeted studies for protein analysis is that the wide dynamic concentration range of proteins in biological fluids or tissues causes many detection difficulties due to a large number of proteins that are below the level of sensitivity of the most advanced instruments. For this reason, protein fractionation that exploits the different physicochemical properties of proteins and subsequent concentration of the selected protein is commonly needed.

In a series of studies, Fernandez Ocaña *et al*. [(Fernandez Ocaña *et al*, 2007); (Fernandez Ocaña *et al*., 2009)] demonstrated the potential of two different MS-based approaches to detect and characterize the transgenic protein CP4 EPSPS in several crops. This recombinant CP4 EPSPS protein confers resistance to the herbicide glyphosate in several commercial GM crops; namely, soya and maize. In the first study, different fractionation and enrichment approaches were used to overcome the interference generated by the abundant seed-storage proteins on the MS detection of the low-abundance proteins (Fernandez Ocaña *et al*., 2007). Gel-filtration chromatography (GFC) followed by SDS-PAGE fractionation was used for CP4 EPSPS protein purification. The authors also observed that an additional anion-exchange prefractionation step after GFC and SDS-PAGE provided a further protein enrichment to allow the analysis of lower levels of CP4 EPSPS protein in the different crop samples. The MS analytical strategy was based on the tryptic digestion of the purified CP4 EPSPS protein of the GM and non-GM crop and subsequent analysis with either MALDI-TOF MS or nLC-ESI-QTOF MS. The methodology permitted the detection of 0.9% GM soya seeds. Furthermore, as the same group demonstrated later, the use of stable-isotope-based MS analysis was an interesting alternative for the target analysis of the transgenic protein (Fernandez Ocaña *et al*., 2009). In that latter work (Fernandez Ocaña *et al*., 2009), the authors investigated the suitability of two different approaches, [namely, the automated quantitative

analysis (AQUA™) system, and the aforementioned isobaric tags for relative and absolute quantification (iTRAQ)] for the absolute quantification of different CP4 EPSPS protein levels in herbicide-tolerant GM soya seeds. The analytical procedure used also a previous fractionation step based on CP4 EPSPS enrichment by combining anion-exchange chromatography and SDS-PAGE in order to reduce the sample complexity. In the AQUA strategy, the heavy isotope-labeled internal standard peptide (L*)AGGEDVADLR (L*=13C), the same amino acid sequence to that of the peptide that originated from the enzymatic hydrolysis of the CP4 EPSPS protein, was introduced into the CP4 EPSPS protein-enriched sample; that mixture was next subjected to tryptic digestion. After nLC-ESI-QTOF MS analysis, quantification was accomplished by comparing signal intensities of the intact native and synthetic peptides, as can be seen in Figure 5. Alternatively, isobaric reagents were used for CP4 EPSPS quantitation. After protein purification and subsequent digestion, the peptide mixture submitted to iTRAQ labeling was fractionated with SCX chromatography before nLC-ESI-QTOF MS analysis. AQUA and iTRAQ procedures demonstrated both the potential for quantitative detection purposes of 0.5% GM soybean seeds. Target analysis with nLC-ESI-QTOF MS is useful to study the expression of LHCb1-2, a pea protein, in transformed tobacco plants (Labate *et al*., 2004). The presence of the recombinant protein was investigated at the different plant-organization levels that ranged from the organelles to the tissue and organ levels. Prior to MS analysis, purification of LHCb proteins with sucrose-gradient ultra-centrifugation and SDS-PAGE was needed. After nLC-ESI-QTOF MS analysis of the purified enzyme-digested proteins, the authors did not detect any major difference in the relative amounts of LHCb proteins in the tobacco plants (Labate *et al*., 2004).

B. MS-based approaches to analyze target metabolites.

Although there is not a direct link between genes and metabolites, genetic modifications might be often connected to specific metabolism responses; e.g., as a result of the activity of given proteins or enzymes. Accordingly, target analysis of metabolites can be useful to study the specific effect produced in an organism by a genetic modification (Villas-Boas *et al*., 2005). This goal is particularly feasible when the desired effect of a genetic modification involves an increase or decrease of a key enzyme within a metabolic pathway that affects the levels of a specific metabolite or a group of metabolites. Similarly, target-metabolite analysis specially applies for the study of the primary effect of the genetic modification in nutritionally enhanced GM crops, or in the so-called second generation GM crops (e.g., those that produce vitamins and other food supplements). MS analysis is helpful as, for example, in a recent investigation on the expression of a tobacco anthranilate-synthase gene introduced in GM soybeans with the aim to generate tryptophan (Trp)-enriched soybeans (Inaba *et al*., 2007). The isoforms expressed in GM soybeans are regulatory enzymes in tryptophan biosynthesis. However, this particular isoform was not sensitive to feedback control by the end-product Trp. To evaluate the effect of the insertion of the transgene driven by the constitutive CaMV 35S promoter on the levels of amino acids in different soybean transformants, GC-MS was successfully used. The MS detector equipped with a classical electron ionization (EI) source and operated in the single-ion monitoring (SIM) mode and a *m/z* range between 50-300 provided the detection of all free amino acids except for arginine; that study demonstrated that GM soybean contained about six-fold as much Trp as the non-modified soybean used as control. In a different study of rice transformed with the same genetic modification, LC-MS/MS was used to analyze free and conjugated forms of indole-3-acetic acid (IAA), a plant hormone derived from the Trp biosynthetic pathway (Morino *et al*., 2005). The analyses

indicated that, in addition to high Trp levels, free IAA and its conjugates were both increased in the transgenic rice; those data suggested that the activity of the recombinant protein or the concentration of Trp (or both) is an important regulating factor of IAA biosynthesis.

MS analysis of target metabolites has also proven to be particularly helpful to develop and characterize GM plants with interesting traits for human health; for instance, GM plants developed to accumulate a metabolite or family of metabolites with a beneficial biological activity. The production of lignans in wheat has attracted attention because these phenylpropane dimers have been associated with anti-tumor activities in animal models. Ayella *et al*. genetically transformed wheat cultivars with a pinoresinol lariciresil reductase gene of *Forsithia* fused to an ubiquitin maize promoter in order to overexpress the enzyme and, therefore, to enhance lignan biosynthesis (Ayella, Trick & Wang, 2007). The LC-MS analysis used to determine the lignan content in transgenic wheat transformants was essential to corroborate and evaluate the functional transformation success and, therefore, the intended effect of the genetic modification. HPLC separations of lignan extracts, obtained from solvent extraction from wheat seeds, were achieved with a C18 column with an ACN-water gradient. ESI-MS (positive mode; from *m/z* 100 to 1500) detected increased levels of secoisolariciresinol diglucoside in transgenic wheat lines to confirm a strong enhancement in lignan levels (Ayella, Trick & Wang, 2007).

GM tomatoes with increased flavonoid glycosides levels is another example of GM crops developed to confer beneficial biological activity to the consumer. In this case, the intended modification brings about some prevention of cancer and other pathologies, because the antioxidant activity of the vegetable has been improved. [(Le Gall *et al*., 2003a); (Le Gall *et al*., 2003b)]. Transgenic tomatoes were generated for the simultaneous overexpression of two maize regulatory genes of flavonoid biosynthesis, *leaf color* and *colorless* [(Le Gall *et al*., 2003a); (Le Gall *et al*., 2003b)]. A variety of analytical techniques (LC with DAD, NMR, MS

and MS/MS) were used to investigate flavonoid composition of transgenic and control tomatoes. The chromatographic analyses of a number of tomato extracts indicated the presence of 7 flavonoids at much higher concentration (up to 60-fold difference) in transgenic tomatoes than in the non-transgenic controls at different stages of maturation. LC-MS and LC-MS/MS data confirmed the identity of the aglycon moiety of two minor, but important, dihydrokaempferol hexosides. This identification was achieved by comparing the main fragmentations of MH^+ ions of the unknown analytes with MH^+ ions obtained from standards of flavonoid glycosides [(Le Gall *et al*., 2003a); (Le Gall *et al*., 2003b)].

The accurate determination of a metabolite or group of metabolites in the different plant tissues and organs is essential in many studies of GM plants. This aspect is especially important in transgenic crops with bioremediation traits (i.e., transgenic crops used to return the natural environment altered by contaminants to its original condition). For instance, in phytoremediation of metals by transgenic plants, the capabilities of the GM plant to hyperaccumulate, transport, or transform inorganic contaminants in the different organisms are relevant aspects that must be studied in order to evaluate the plant potential to assist in the remediation of metal-contaminated soils. For efficient soil remediation, phytoextraction must be coupled to translocation to other plant tissues that are more readily accessed and removed. The translocation of metals can be studied by LC connected to inductively coupled plasma mass spectroscopy (LC-ICP-MS) as it is illustrated in a recent study aimed at the development and application of an analytical method for the accurate determination of cadmium-phytochelatins in *A. thaliana* plants that are genetically modified to express the wheat phytochelatin synthases under the control of the constitutive CaMV 35S promoter (Sadi *et al*., 2008). To facilitate the speciation of cadmium-phytochelatin complexes, the high efficiency and resolution capability of LC was combined with the excellent sensitivity of ICP-MS for cadmium-selective detection. The main advantages of LC-ICP-MS are derived from its use

for speciation analysis, however, prior to method development, an ICP-MS interface was modified in order to improve the sensitivity for the analytes that elute in high organic solvent mobile phase from the RP-HPLC column. These modifications included the use of solvent-resistant materials, the use of vapor-pressure control by cooling the spray chamber to avoid plasma disruption, and the addition of 5% oxygen (v:v) to the Ar carrier flow to react with the carbon in the sample to prevent any carbon deposition on the sampling cone. Parameters that affect the resolution of the analytes with a C18 HPLC column, mobile phase composition and pH, were investigated. The method provided limits of detection (LOD) for three different cadmium-phytochelatin complexes that ranged from 49 to 92 ng/L in plant extracts. Analyses, under optimized RP-HPLC-ICP-MS conditions, of root and shoot extracts from *Arabidopsis* plants grown in the presence of 10 μM Cd^{2+} demonstrated that cadmium (as cadmium-phytochelatin complexes) accumulation was greater in the root and the shoot tissues of the transgenic plant as compared to the wild type. Also, the higher amounts of cadmium complexes found in the shoots than in the roots suggested that phytochelatins contributed to cadmium accumulation in aerial parts of the plants.

CE-MS coupling was used for the first time by Bianco *et al*. in 2003 for the analysis of target metabolites in GM crops (Bianco *et al*., 2003). The study was aimed to the analysis with a previously optimized method based on NACE-ESI-IT MS coupling (Bianco *et al*., 2002) of glycoalkaloids (GAs), known antinutrient compounds, in tubers of GM virus Y-resistant potato plants . The use of organic solvents with volatile electrolytes for the CE separation of GAs exhibited very good MS compatibility. The GA content was evaluated from methanolic extracts from three lines of modified potato plants (var. Désirée) resistant, intermediate, and susceptible to infection by potato virus Y (PVY) and a conventional cv. Désirée. IT MS/MS identified α-chaconine and α-solanine GAs. It was found that the highest level of total GAs was found in the peel; potato tubers from the resistant line showed a

slightly higher content of α-solanine in the peel and in the flesh when compared to tubers of control (Bianco *et al*., 2003).

IV. FUTURE OUTLOOK

Crop (and plant) proteomics is a growing discipline. The full potential of proteomics has not yet been exploited in plant research when compared with other proteomic technologies used to investigate prokaryotes, yeast, and humans. 2-DGE, together with MS-based methodologies, remains the most widely available approach for proteomic analysis of GM crops, even though there is a notorious difficulty to reproduce results between laboratories. An on-line combination of chromatography and/or capillary electromigration methods, coupled to advanced MS instruments, has already been demonstrated to be effective in the analysis of proteins and peptides to create new and encouraging perspectives to address the discovery of any proteome.

New generation MS instrumentation provides increasingly accurate qualitative and quantitative data on proteomics and metabolomics. New compact mass analyzers, such as the Orbitrap, provide high mass accuracy with the possibility of MS/MS experiments and less cost than FT-ICR-MS. Also, the Orbitrap, which offers new perspectives for proteome and metabolome analysis [(Yates *et al.*, 2006); (Macek *et al*., 2006); (Kiefer, Portais & Vorholt, 2008)], has still not been demonstrated in the analysis of GM crops. It is also expected that high-resolution hybrid mass spectrometers, such as a Q-TOF MS instrument, with a proven suitability for metabolic profiling, will find more applications in the near future within the GMO research field (Goodacre *et al*., 2002). Also, developments such as nano-ESI MS with better sensitivity capabilities can be used to improve metabolomic MS analysis in plants by

direct infusion [(Sudjaroen *et al*., 2005); (Lokhov & Archakov, 2009)]; therefore, their use for GM plant investigations is also predictable. Improvements in coupling MS with separation or other analytical techniques must also be considered. Analytical platforms that combine conventional chromatographic/electrophoretic techniques with MS are capable of reasonable resolution and moderate throughput. However, higher resolution and peak-capacity alternatives to conventional couplings for complex protein and metabolite profiling offer good possibilities for GMO characterization. Among these techniques, UPLC-MS and nano-LC-MS have already demonstrated excellent capabilities in the analysis of metabolites [(Muth et al, 2008); (Lohkov & Archakov, 2009)]. In this regard, the hyphenation of CE with MS will achieve all its huge potential once more-robust, -sensitive, and -reproducible interfaces become available for this coupling. Also, one of the most promising alternatives to conventional MS platforms for metabolomic studies of GM crops is comprehensive two-dimensional chromatography (GCxGC and LCxLC). Thus, comprehensive two-dimensional GC-MS (GCxGC-MS) is ideal for the analysis of volatile compounds in complex samples. However, GCxGC requires very high-scan speed mass analyzers. Accordingly, the good scanning speed of TOF-MS detectors, as well as its high resolution and mass accuracy, makes them the best option for this coupling (Herrero *et al*, 2009). This coupling has already been used for analysis of compounds in plants (Pierce *et al*, 2006) and foods like carrots, tea, or berries [(Dallüge *et al*, 2002); (Schurek *et al*, 2008); (Banerjee *et al*, 2008)]. Comprehensive two-dimensional LC-MS (LCxLC-MS) is also a potential tool for metabolite analysis in plants and foods, as already demonstrated [(Dugo *et al*., 2008); (Mondello *et al*., 2008)].

As mass spectrometry evolves with more powerful and complex instruments, more data are generated that must be analyzed. Data processing is already a critical step to obtain a successful "-omic" study, and represents a new challenge in the bioinformatics field. Accordingly, computational techniques must be developed to process, integrate, and analyze

MS data. A precise integration of a wide range of analytical methodologies with MS as well as the coordination of multiple scientific specialists (from biological sciences, separation sciences, mass spectrometry, and bioinformatics) is still needed to characterize entire proteomes and metabolomes. In this regard, the development of global data-integration approaches (like, e.g., systems biology) will provide a comprehensive and deep knowledge on the entire process taking place in any organism (i.e., from the DNA level to the final metabolite, passing through the generated transcript and protein).

Data collection from different laboratories will increase proteome and metabolome coverage, and improve present proteome information from non-model or poorly characterized crops. However, the quality of the proteomic and metabolomic data, as well as the quality of the experimental design and proper statistical data treatment, should be improved in future studies. Also, more work is needed to characterize natural variability of crops to make easier the identification of any unintended effect or GM crop. The definition of common standardized experimental protocols is a major challenge in proteomics and metabolomics. Unifying analytical platforms and protocols will allow the comparison of experiments performed in laboratories worldwide. In addition, much effort is needed to integrate proteomics and metabolomics with genomics data. This integration will involve a vast quantity of collaborative work to compare and share data within the scientific community. All of these advances will serve to determine common proteomic and metabolomic workflows in order to apply these MS-based methodologies as part of the existing comparative safety assessment process for GM crops.

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Table 1. Study of the substantial equivalence of GM crops with MS-based proteomic profiling methods.

Crop	Donor organism	Protein expression	Trait/phenotype	Tissue	Protein separation	Protein identification	Ref.
Tomato	Tomato spotted wilt virus	TSWV nucleoprotein	Virus resistance	Seed	2-DGE	MALDI-TOF MS	Corpillo *et al*., 2004
	-	ScFv (G4)	Virus resistance	Leaf	DIGE	MALDI-TOF MS, µLC-ESI-IT MS/MS	Di Carli *et al*., 2009
Tobacco	-	ScFv (B9)	Virus resistance	Leaf	DIGE	MALDI-TOF MS, µLC-ESI-IT MS/MS	Di Carli *et al*., 2009
	Tomato	prosystemin	Insect resistance	Leaf	2-DGE	MALDI-TOF MS, µLC-ESI-IT MS/MS	Rocco *et al*., 2008
Maize	B.thuringiensis	CryIA(b)	Insect resistance	Seed	2-DGE	MALDI-TOF MS	Albo *et al*., 2007
	B.thuringiensis	CryIA(b)	Insect resistance	Seed	2-DGE	nLC-ESI-IT MS/MS	Zolla *et al*., 2008
Wheat	Tobacco	Rab1	Improved functional properies	Seed	2-DGE	MALDI-TOF MS, nESI-QqTOF MS/MS	Di Luccia *et al*., 2005
	Wheat	LMW-GS	Improved functional properies	Seed	2-DGE	LC-ESI-QTOF MS/MS	Scossa *et al*., 2008
Potato	Potato	(Antisense G1-1 gene)	Sprouting delay	Tuber	-	MALDI-TOF MS	Careri *et al*., 2003
	Aureobasidium pullulans	Glucan branching enzyme	Waxy phenotype	Tuber	2-DGE	µLC-ESI-IT MS/MS, µLC-ESI-QqTOF MS/MS	Lehesranta *et al*. 2005
	Potato	Glycoprotein	Changes in cell wall structure	Tuber			
	Potato	AdoMetDC	Modified metabolism	Tuber			

Table 2. Applications of MS-based approaches for metabolite profiling of GM crops.

Crop	Donor organism	Protein expression	Trait/phenotype	Tissue	Technique	Ref.
Rice	*B.thuringiensis*	Bt toxin, skc protein	Insect resistance	Grain	GC-EI-Q MS	Zhou *et al*., 2008
Raspberries	Dwarf virus	Virus movement protein	Virus resistance	Fruit	GC-EI-Q MS	Malowicki, Martin & Qian, 2008
Soybean	*Petunia hybrida*	EPSPS enzyme	Glyphosate tolerance	Grain	GC-EI-Q MS	Bernal *et al*., 2007
Maize	*B.thuringiensis*	Bt toxin	Insect resistance	Grain	GC-EI-Q MS	Bernal *et al*., 2007
Cucumber	*Thaumatococcus daniellii*	Thaumatin-II	Sweet flavour	Fruit	GC-EI-Q MS, GC-TOF MS	Zawirska-Wojtasiak *et al*., 2009
A. annua	*A. annua*	Farnesyl diphosphate synthase	Artemisinin increase	Leaf	GC-EI-Q MS	Ma *et al*., 2008
Poplar	Poplar	PttPME enzyme	Methylesterification of homogalacturonan	Xylem	GC-TOF MS	Wiklund *et al*., 2008
Tomato	*A.thaliana*	Hexokinase	Altered carbohydrate metabolism	Leaf and fruit	GC-EI-Q MS	Roessner-Tunali *et al*., 2003
	Potato	Modified starch & sucrose metabolism	Altered starch composition	Tuber	GC-EI-Q MS	Roessner *et al*., 2001a
Potato	*Aureobasidium pullulans*	Fructokinase, α-glucosidase, tetracycline, S-adenosylmethionine	Starch biosynthesis, leaf morphology, ethylene production	Tuber	GC-EI-Q MS	Shepherd *et al*., 2006
	Artichoke	1-SST, 1-FFT proteins	Inulin synthesis	Tuber	GC-TOF MS, LC-Q MS	Catchpole *et al*., 2005
Tomato	Grapevine	STS enzyme	Resveratrol synthesis	Fruit	LC-ESI-Q MS	Nicoletti *et al*., 2007

Wheat	Barley	B-1,3-glucanase	Antifungal activity	Leaf	LC-ESI-IT MS/MS	Ioset *et al*., 2007
Grapevine	*E.coli*	Adehide dehydrogenase	Abiotic stress	Leaf	LC-ESI-IT MS	Tesniere *et al*., 2006
Rice	Tobacco	Anthranilate synthase	Nutritionally enhanced	Calli	LC-ESI-Q MS/MS	Morino *et al*., 2005
	Maize	C1 & R-S regulatory genes	Flavonoid production	Leave	LC-ESI-IT MS/MS	Shin *et al*., 2006
Rice	Maize	Dihydroflavonol-4-reductase	Stress tolerance	Plant and calli	CE-ESI-MS	Takahashi *et al*., 2006
Maize	*B.thuringiensis*	Bt toxin	Insect resistance	Grain	CE-ESI-TOF MS	Levandi *et al*., 2008
Soybean	*B.thuringiensis*	Bt toxin	Insect resistance	Grain	CE-ESI-TOF MS	Giuffrida *et al*., 2009
	P. hybrida	EPSPS enzyme	Herbicide tolerance	Grain	CE-ESI-TOF MS	García-Villalba *et al*., 2008
Maize	*B.thuringiensis*	Bt toxin	Insect resistance	Grain	FT-ICR-MS	Leon *et al*., 2009
Rice	Maize	YK1 protein	Stress tolerance	Calli and leaf	FT-ICR-MS	Takahashi *et al*., 2005
Tobacco	*E. coli & S. hygroscopicus*	NADPH-GDH enzyme	Water deficit tolerance	Seed	FT-ICR-MS	Mungur *et al*., 2005

FIGURE LEGENDS

Figure 1. Schematic flow diagram of the different bottom-up strategies for protein analysis from complex protein extracts using MS-based analytical technologies.

Figure 2. GC-MS total ion chromatogram of different tissues from conventional tomato. A, GC-MS from tomato leaf; B, from fruit after 30 days maturation and C, fruit after 60 days maturation. From Roessner-Tunali *et al*, 2003.

Figure 3. CE-TOF-MS extracted ion electropherograms of the metabolites detected in two maize varieties; a transgenic one (PR33P66 Bt) and its corresponding isogenic line (PR33P66). Both varieties were grown in the same conditions. From Levandi *et al.*, 2008.

Figure 4. Partial least-squares discriminant analysis (PLS-DA) and Van Krevelen diagrams (atomic H/C versus O/C) generated from FT-ICR-MS analysis of maize extracts obtained with PLE and three different solvents (hexane, methanol, and water). For the three different groups (different solvents) the masses with the highest coefficient of regression are considered. These masses are represented in Van Krevelen diagram, and were submitted to MassTRIX reveal that the main differences between hexane and water consist in the massive presence of carbohydrate metabolism in the water and fatty acid in the hexane. From León *et al*., 2009.

Figure 5. LC-TOF MS extracted ion chromatograms from 47 kDa SDS-PAGE of anion exchange fractions from (A) 0.5%, (B) 0.9%, (C) 2%, and (D) 5% GM soya. $[M+2H]2+$ = *m/z* 558.30 corresponds to CP4 EPSPS tryptic peptide LAGGEDVADLR. $[M+2H]2+$ = *m/z* 561.30 corresponds to synthetic peptide (L*)AGGEDVADLR. From Fernandez Ocaña *et al*., 2009.