Plant Metabolomics: Missing Link in Next Generation Functional Genomics era

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ABSTRACT

Metabolomics plays significant roles in plant biology including growth, development and stress resistance. Plant produces diverse array of metabolites (approximately 200,000 to 1,000,000); hence metabolomic study is of great importance in plant biology. Due to presence of diverse array of metabolites in plants, it posses greatest challenge to indentify and quantify them correctly. Very significant improvement has been made in the field of plant metabolomics, but uniform annotation of metabolite signals in database and informatics of international standardization remains a challenge. The advancement of metabolomics largely depends upon increase in separation efficiencies and identification of individual metabolites. Fluxome and metabolomic QTL (mQTL) are very important missing link in plant metabolomics. Now these days, metabolomics is a part of system biology and metabolomics in combination with system biology approach will lead to unbiased acquisition of mass spectrometric data from diverse array of samples. To overcome different challenges, development of improved technology for detection and identification of metabolite in complex plant tissue and dissemination of metabolomic research data will be very helpful.

1. INTRODUCTION

The primary aim of the "OMICS" technology is non targeted identification of all gene products (transcripts, proteins and metabolites) [1]. After the establishment of technologies for high-throughput DNA sequencing (genomics), gene expression analysis (transcriptomics), and protein analysis (proteomics), the remaining functional genomics challenge is that of *metabolomics* [2,3,4].

Metabolism is the term coined for essentially comprehensive, non biased, high-throughput analyses of complex metabolite mixtures typical of plant extracts [5,6,7,8]. This potentially holistic approach to metabolome analysis is driven primarily by recent advances in mass spectrometry technology and by goals of functional genomics research [9,10,11].

Achieving the broadest overview of metabolic composition is very complex and entails establishing a multifaceted, fully integrated strategy for optimal sample extraction, metabolite separation, detection, automated data gathering, handling, analysis and ultimately quantification [12,13]. Both analytical and computational developments are essential to achieve this goals.

2. METABOLIC DIVERSITY AND DATABASE

Human knowledge so far encompasses the existence of at least 270,000 plant species [14], and researcher believe that more than 400,000 plant species exist worldwide. The total number of metabolites in plant kingdom is estimated to range from 200,000 to 1,000,000 [15]. The metabolic richness comes not from the number of genes present (20,000 to 50,000) but also from multiple substrate specificities for many enzymes [16].

Therefore large scale comprehensive metabolite profiling meets its greatest challenge-a challenge that provides the impetus cutting edge technological development. Various experimental approaches are currently being pursued to profile and determine the chemical identity of plant metabolite [14]. The analytical approaches deployed vary in their relative concentration and chemical complexities [17,18]. Typically metabolites are identified through spectral comparison with authenticated compounds contained with spectral libraries [14]. Some of the commonly used spectral libraries are the NIST (www.nist.gov), Wiley (http://wileyregi stry.com), or Sigma-Aldrich (www.sigmaal drich. com) libraries. Although these libraries contain more than 350000 entries, most of these are non biological complex and lack information on chromatographic behavior [19].

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Tokimatsu *et al.,* (2005) reported an web based analysis tool known as KaPPA-view for integration of transcript and metabolite data on plant metabolic pathway maps [20]. Metabolomic research   will prove an invaluable tool for generating information of special importance in many fields [21]. For functional genomics strategies, potentially fast-track methods exploiting metabolomics analyses of tagged lines or unknown mutants are likely to provide invaluable information's [22]. Metabolomic information will not only assist in the establishment of deeper understanding of complex interactive nature of plant metabolic network and their response to environmental and genetic change but also will provide unique insight into the fundamental nature of plant phenotypes in relation to development, physiology, tissue identity, resistance, biodiversity etc. [23,24]. Currently metabolomics being applied in many biological studies ranging from carbon-nitrogen interactions in plants to development of personal metabolomics as next generation nutritional assessment in humans [25]. A better understanding of the correlation between genes and functional phenotypes of an organism will be the true goal of all functional genomics strategies.

3. PRESENT STATUS OF METABOLOMICS

It is essential to follow the response of an organism to a conditional perturbation at the transcriptome, proteome, and metabolome level [26,27]. These three levels of expression profiling provides a complete picture of RNA's protein and metabolite that enable one to infer relevant association between macromolecules; identify functional linkage between phenotypic expressions; and construct model that quantitatively describe the dynamics of biological system [28,29,30]. Broad phenotypic analyses are very essential if we have to progress from prediction to experimental validation of gene function [17].

Plant metabolites functions in different stress response and undergoes resistance mechanism against different biotic and abiotic stress agents [31]. These metabolites are act as antibiotic, anti-feeding, antioxidant, anti-herbivory agent [32]. These also contribute to color, taste, aroma, and scent of flowers and fruits [33]. The metabolic phenotype of an organism is the final result of interactions between genotype and environment [34,35,36,37], but it also modulated by sub-cellular physiological fluctuations that are part of homeostasis [1]. Thus, the simultaneous identification and quantification of metabolites is necessary to study the dynamics of metabolome [19], to analyze fluxes in metabolic pathways and to decipher the role of each metabolite following various stimuli [34]. The challenges of metabolomics is to find changes in the metabolomic network that are functionally correlated with physiological and developmental phenotype of a cell, tissue or organism [38]. Linkage of functional metabolomic information to mRNA and protein expression data makes it possible to visualize the functional genomics repertoire of an organism [39,40,41]. This knowledge has great potential for application in efficient engineering of crops that combines an attractive appearance and taste with improved level of phytonutrients like flavonoids and carotenoids [2,42].

4. METABOLITE PROFILING TECHNIQUES

The huge diversity of chemicals produced by plant is due to the diverse functions of the genes of their genomes [43]. Metabolomics is the rapidly emerging field of post genomic research [14]. A metabolome represents the ultimate phenotype of cells deduced by perturbation of gene expression and the modulation of protein functions [44,45,46]. In addition, the metabolome can also influence gene expression and protein function [27,47,48,49]. Therefore metabolomics plays a key role in understanding cellular system and decoding the functions of genes [26,44,50].

The large variations (-10^6) in the relative concentrations of metabolites also make metabolite analysis more complicated. Therefore, comprehensive coverage can only be achieve by using multi-parallel complementary extraction and detection technologies with careful experimental design [14]. Currently there is no single technology available to detect all the compounds in single analysis in any plants or other organisms [50,51,52], a combination of multiple analytical techniques, such as gas chromatography (GC), liquid chromatography (LC), capillary electrophoresis (CE)-MS, NMR, and rapid scanning time-of-flight (TOF) are generally performed to detect maximum compounds [10,34,53,54].

4.1 General chromatographic procedure:

Metabolites can be extracted from few milligram of tissues. First of all to grind them to make them fine powder and extraction can be carried out by either in polar/semipolar (e.g. methanol/water or ethanol/water) or non polar (e.g. chloroform) solvent. Volatile components can be extracted with solvent or headspace extraction [55].

Samples for instrumental analysis can be prepared from the crude extract by partial purification method such as liquid-liquid extraction and solid phase extraction. In case of GC-MS (gas chromatography-mass spectroscopy) method, a two steps derivatization by methoxyamination and trimethylsilylation represent a key techniques for profiling of hydrophilic metabolites [56].

Metabolic profile data can be obtained by using GC-MS, LC-MS and NMR [57].

Metabolite signals in row chromatogram/spectrum data need to detect and quantify comprehensively. These data need to assigned with metabolite information to produce a data matrix listing a metabolite and its intensity data. The peak picking process which was a technical bottleneck in the early stage of metabolomics study can now be performed routinely by developing a series of software dealing with GC, LC-MS, CE, and NMR data in which matrices containing intensity values of all signals in sample are taken into consideration [56,58,59].

The metabolic profile data then can be analyze by using various data mining techniques [60,61]. Most technology for metabolomics is based on mass spectrometry [62,63]. Gas chromatography– MS (GC-MS) and HPLC–photodiode array–MS remain the methods of choice for quantitative and qualitative metabolite profiling [64,65,67,68]. The ultimate goal of metabolomic is ability to comprehensively detect and quantify every metabolite in a plant extract, is unlikely to be attained by any single analytical method available at present. LC-MS is an unique method for profiling plant secondary metabolite like phenylpropanoids and alkaloids [62,63,69]. One of the important mission of LC-MS analyses in metabolomic study in plant research is to understand phytochemical diversity within the plant [70,71,72,73].

Rapid scanning time-of-flight (TOF) MS coupled with GC separation and integrated with peak deconvolution software technique increased the number of metabolites detectable by GC-MS in crude plant extracts to 500 to 1000 [64,74]. However, the dynamic range of TOF detectors is still restrictive when faced with mixtures containing compounds with concentration differences of several orders of magnitude. TOF and FT (fourier transform)/MS have been employed to obtain elemental composition data of each metabolite signal [30,63]. CE-MS being employed to detect ionic metabolite such as cation, anion, nucleotides, sugar phosphates, organic acids, amino acids [75,76].

5.FALSE DISCOVERIES IN METABOLOMICS

There are two possible classes, the outcome of any predictions relative to the 'true' class membership is usually set out as a binary matrix, the so-called confusion matrix consisting of true positives (TP), false positives (FP), true negatives (TN) and false negatives (FN) [77,78]. The term false discovery rate (FDRs) typically refers to the frequency of type I errors, i.e. to claims that some variable, or model, can discriminate two population when, in fact, it can't [79,80,81]. One of the main cause of FDRs is, inadequate sample size [82,83,84], a features that is particularly problematic in cases when the number of variables greatly exceeds the number of samples. Many metabolomics and other high-throughput experiment are set up in such a way that the primary aim of the discovery of biomarker metabolites can be discriminate with a certain level of certainty between normally matched 'case' and 'control' sample [85,86]. However, it is unfortunately very easy to find biomarkers that apparently persuasive but that are in fact entirely spurious.

The main type of danger are not entirely depends upon each other, but include bias, inadequate sample size (especially relative to the number of metabolite variables and to the required statistical power to prove that a biomarker is discriminant), excessive false discovery rate, due to multiple hypothesis testing, inappropriate choice of particular numerical method, and over fitting (generally caused by the failure to perform adequate validation and cross validation) [87,88]. Many studies fails to take these into account, and thereby fail to discover anything of true

significance (despite of their claim). The world of science is littered with samples of false conclusion being drawn from ostensibly well designed experiments, and the bad design of experiments will usually ensure such an outcome. In many areas of post genomic discovery, the proper methods of statistical analysis are not entirely clear, for instant how best to treat correlated variables in terms of Bonferroni-type correction for significance when doing multiple hypothesis-testing [89,90]. With very many variables, potentially with significant noise, the false discovery rates and premature claims of significance are likely to be major problems [91,92].

6. THE MISSING LINK

6.1 Fluxome

An additional layer of omics is "Fluxome", is required between proteome and metabolome layers to consider the dynamic aspects of plant metabolism [51,92]. These are steady-state rates of metabolic inter conversion within living cells [93]. They constitute a significant aspect of cellular phenotype, determining the rates of growth and product formation. Flux balance analysis widely used in calculating steady-state flux distribution. The principle of flux-balance analysis relies on experimentally quantifying a set of metabolic fluxes in the network; such as substrate consumption or product excretion. One disadvantage of flux-balance analysis is that, it is unable to predict quantitatively to what extent certain enzyme activities should be altered to achieve a desired effect, such as an increase in the specific production rate of a product. Zhu *et al.,*(2012) developed thermodynamic optimum searching (TOS) to improve the prediction accuracy of flux balance analysis [94].

Fluxome is also called metabolic regulon which impairs control of metabolite level via regulation of metabolic flux of the pathway responsible for biosynthesis and catabolism [93,94,95,96]. Thus a quantitative investigation of metabolic flux can give us more deep understanding of modes of regulation of plant metabolic system [97]. Up and down regulation of metabolic flux can be estimated qualitatively from the expression level of gene and enzyme assay [96]. By using the enzyme assay platform, activities of metabolic pathway can be elucidate more directly than with transcriptome data [98], since the "net" activity of each reaction step, including multiple isoenzymes can be determined. Most direct information of metabolic regulon can be obtain by determination of an actual metabolic flux [92,99]. Dynamic labeling of ^{13}C isotope provided to be powerful tool to elucidate metabolic regulon mechanism [99,100]. The dynamic labeling technique enables the metabolic flux from kinetics of *in vivo* isotope labeling [101]. By using this techniques, various dynamics aspects of plant metabolism have been investigated [102, 103]. Rossell *et al.,*(2011) presented a method by which flux changes can be predicted using enzyme concentration changes only. They thought reaction rate and enzyme level are proportional and determines the steady-state fluxes and complexity of enzyme-metabolite interaction is secondary [93].

6.2 Metabolome QTL

Quantitative trait locus mapping of metabolic phenotypes (mQTL) is a powerful approach to unravel the genetic component associated with metabolic profiles and identifies genes associated with metabolic marker of disease. The mQTL mapping constitutes genetically heterogeneous cohort, modern genotyping platform, hypothesis-free metabolic profiling using high throughput nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS), generating up to 20,000 metabolic trait per sample and the statistical tool required to map these trait on to the genome of experimental population. Network and system biology strategy can enhance the biological interpretation derived from mQTL studies.

Most agriculturally important traits are under the control of quantitative trait loci [104, 105,106,107]. Metabolite level of plant tissue (m-trait) are also a quantitative trait. Recently metabolome QTL (mQTL) analysis regarding m-trait have been conducted for comprehensive understanding of their genetic background [40,41,108,109]. A number of causal genes, responsible for mQTL can be deduced by high density linkage map of molecular markers and these can be taken for further investigation of regulatory system in complicated plant metabolism pathway.

Khan *et al.,*(2012) reported mQTL hotspot for phenolic compound of apple (*Malus X domestica* Borkh) in linkage group 16 [110]. Untargeted mQTL mapping of metabolites showed 669 mQTL in peel and flesh, spread over 17 linkage group of apple genome. Not all the metabolites showed mQTL and 99 annotated metabolites were belonged to phenolic compounds (phenylpropanoids and polyphenols). The genetic loci controlling metabolite content in peel also control the flesh of apple in less significance manner. The level of metabolites in the linkage group LG16mQTL hotspot controlled by a single, dominant locus present in both parents [110]. Flavonoid mQTL hotspot is located on LG16 and the quercetin glycosides are not controlled by flavonoid mQTL hotspot on LG16 and no mQTL was detected for many metabolites.

The mQTL analysis of *Arabidopsis thaliana* revealed that QTLs are unevenly distributed in the genome [40], and there are several regions where the densities of QTLs are much higher than in other region [30, 40]. Although it never been elucidated whether the mQTL hotspots are derived from a variation of only one key gene or that of gene clusters [111,112], the existence of QTL hotspot suggests that the overall composition of plant metabolome can be controlled by the manipulation of small genomic region [14]. The relationship between mQTL trait and other important agronomic and biological trait like taste, yield, biomass etc. can be investigated as these traits likely interact closely with metabolism in plants [40, 113,114]. Metabolome analysis of tomato fruit indicated that there are certain correlation among these traits [37,109]. Regression analysis of metabolome data for *Arabidopsis* biomass trait demonstrated that growth rate of *Arabidopsis* seedling is some extent predictable from the metabolome signature [113].

7. CURRENT LIMITATION OF METABOLOMICS AND FUNCTIONAL GENOMICS APPROACH

Advancement of metabolomics depends upon increase in separation efficiencies and identification of individual metabolites. Unlike mRNA and protein, it is difficult or impossible to establish a direct link between individual metabolites and its corresponding gene. Functions have been proven for many plant metabolites or can be inferred from our knowledge of other organisms [115,116]. However a same metabolite can be member of several different pathways and can also have regulatory effects on unambiguously linked to a single genomic sequences [45].

No longer, metabolomics seen just as a tool of functional genomics, but it has now became an integral part of system biology [3]. Early approaches were based on metabolite fingerprints and metabolite profiles, but there are also studies focusing on fluxes. The array of techniques to measure a large number of metabolites has also been expanding. The application of metabolomics currently limited by computational issues. The biggest metabolomic challenges posed by metabolomics are (1) to identify large number of metabolites that are detected but, whose chemical nature is unknown (estimates goes from 60% to 90% of total) (2) to identify the active areas of metabolism (pathways and networks) responssible for changes in metabolite profiles and (3) to create standards for data and metadata format and reporting [3]. It is unambiguously important to create a data standard that cover all of metabolomics and will be compatible with other standards of genomics and proteomics. Simultaneous application of metabolomics, proteomics, and transcriptomics that are likely to provide the most comprehensive and informative views of biological systems, for this, it is crucial that we must have data standards in place. The next step will be to integrate these data in global models of cellular behavior-indeed a truly system view of biology.

8.APPLICATIONS OF METABOLOMICS TO PLANT SYSTEM

Metabolomics offers the unbiased ability to differentiate genotype based on metabolite levels that may or may not produce visible phenotype [117,118]. Furthermore, in those instances in which mutations or expression of transgenes lead to measurable phenotype changes. Metabolic approaches can be used to decipher the biochemical cause of consequence of the observed phenotypes.

Metabolomics at its most powerful when performed on a large scale and integrated with corresponding data on the transcriptome and proteome. More selective metabolic profiling however has been used in a number of areas to provide biological information beyond the simple identification of plant constituents [18]. These area include Fingerprinting of species, genotypes or ecotypes for taxonomic or biochemical aspects [119].

Monitoring the behavior of specific classes of metabolites in relation to applied exogenous chemical and/or physical stimuli [120]. Studying symbiotic association [121]. Comparing metabolite content of mutant or transgenic plant with

that of their wild type counterparts. Coupling of metabolite profiling with that "omics" technology can provide an integrated picture encompassing all aspects of information flow from genome to metabolome and resulting phenotype.

9. FUTURE DIRECTIONS FOR METABOLOMICS

Development of improved technology is necessary for determination of metabolites in complex plant tissue and dissemination of metabolomics research data [20,122,123,124,125]. To integrate and disseminate metabolomics research data, a metabolomic information standard will help to ensure that metabolite data and meta data can be easily interpreted and that result can be independently verified outside the original source laboratory [126]. Elevating technical performance to enable broader capture of metabolomic data with the required throughput and accuracy of identification is even more challenging. Because these challenges are widely recognized and endorse [127]. This encourages a community based effort to define common criteria and to initiate several concerted actions. Different steps to overcome the challenges:

9.1 Modeling and simulation

Model can be used for prediction and generation of hypothesis [18]. Mathematical modeling of metabolism is a powerful techniques for gaining sufficient quantitative understanding of complex metabolic pathways [128,129] in order to alter the distribution of metabolic flux or to rationally design metabolic pathway for new product [130]. Transient isotopic flux analysis and kinetic modeling are proven to be powerful techniques for quantification of metabolic fluxes in compartmentalized and dynamic metabolic system [128]. These tools are now widely used to address metabolic flux response to environmental and genetic perturbations in plant metabolism. Continuous development in isotopic and kinetic modeling, quantifying metabolite exchange between compartments, and transcriptional and post transcriptional regulatory mechanism governing enzyme level and activity will enable simulation of large sections of plant metabolism under no-steady state conditions [131]. Metabolic control analysis will continue to make substantial contributions to the understanding of quantitative distribution of control of flux [128].

9.2 Improved comprehensive coverage of metabolome

Metabolomic data ultimately relies on accurate identification of metabolites [132]. Scientific literature including elemental analysis, NMR and MS spectral data of isolated compounds are necessary to ensure accurate metabolic identifications [58]. A single chemical shift or mass value is insufficient to provide confident metabolite identification. Therefore definition of a minimum quality standards for metabolite identification in metabolite profiling experiments is necessary. However this is not as stringent as for novel compounds those are identified for the first time, otherwise it is minimum that

molecular mass or mass spectral fragmentation pattern of GC/MS or LC/MS should match apparently. Hyphenated techniques that couples chromatography to mass spectroscopy and/or to NMR, such as LC/MS/NMR analysis might offer the greatest confidence in sample identifications, but represents a large expenses [21, 58,133,134,135]. Single spectrometric determination gives insufficient detail for confident metabolite identification. The best analytical approaches for large-scale screening and preliminary identification of unknown compounds can carry out with two-dimensional instrumental techniques (based on each combination of GC/MS, LC/MS, GC/MS/MS, LC/MS/MS or LC/NMR/MS), which enable both comparative profiling and structural elucidation [135,136]. For example, LC/QTOFMS/MS (liquid chromatographic quadrupole tandem time-of-flight mass spectroscopy) has the potential to provide accurate mass and metabolites [137]. Experimental mass data can then be used for calculating an elemental composition and can be compared with available mass information.

9.3 Reference material and facilitation of comparative result

Standard reference materials would allow comparison of the experimental and instrumental efficacy between laboratories and technologies. Because most metabolomic approaches uses different technology platforms (e.g. Fourier Transform/MS, Time-Of-Flight/MS, ion-trap and NMR) that vary in their range of measured metabolites, accuracy, resolution, dynamic range and sensitivity reference materials would allow validation of technical performance and a mechanism for comparative performance evaluation [59, 70,138,139].

9.4 Integration of metabolomics with other functional genomics data

The development, establishment and integration of metabolomics database will bridge the barriers between metabolomics and other functional genomics approaches (i.e. transcriptomics and proteomics) and will allow the development of biological systems networks by integrating transcriptome, proteome, metabolome and flux data [20,26,49,140,141]. The *Arabidopsis* information resources (TAIR), genomic database of Japan (www.genome.ad.jp /kegg/pathway.html), maize genome database (http://www.maizegdb.org) etc are emerging so rapidly, but it became even more essential to develop com prehensive metabolomics dataset. Maturation of metabolomics as the next milestone of functional genomics ultimately depends on establishing metabolomics relational database, that store, compare, integrate, and enable the determination of causal relationship between genes, transcripts, proteins and metabolites [21,142,143,144,145,146,147]. All functional genomics approaches are "information rich", but each method is also vulnerable to various statistical cavets, because the data originates from different samples characterized by several thousand feature like genes, m/z (mass to charge ratio), spectral intensities etc [78,148]. These might lead to difficulties in interpretation and validation of data. The generation of database tools to query and/or

comprehensively mine metabolomic data depends on availability of metabolite database that can be trusted and for which the source of data and its history are maintained and made publicly accessible. In each data repository, expert assessment and data curation are important to assure the uniformity and quality of the information [149,150].

Data acquisition, transformation, validation and annotation are all aspects of curation. There is not any generally adopted procedure to transform and annotate metabolite data yet been proposed that can be used independently from its technological platform. There is great need for validated data models that defines suitable approaches for generation, processing and storage of metabolomic data. To facilitate data model development, it can be possible through cooperative research that integrates different datasets. Future step will use the validated and curated metabolomic information to study the dynamics of metabolome to analyze fluxes in metabolic pathways and to decipher the biological relevance of each metabolite. As the comprehensiveness increases and bioinformatics tools mature, functional metabolomic information can be linked to transcriptome and proteome dataset to enable a better understanding in plant biology.

10. CONCLUSION

Metabolomics study is particularly important in plant biology field, because plant produces huge array of diverse metabolites. Complete identification and quantification of these metabolites cannot be achieved by single analytical technology, but multi-parallel complementary technology will be very helpful. Transcriptome co-expression network will provide invaluable information to decode co-occurrence principle of transcript and metabolites. So, it can be very helpful to identify evaluation of genetically modified crop.

Metabolomics with system biology will play key role in understanding plant system and hence development of biotechnological applications. Metabolome QTL (mQTL) along with gene expression and agronomical trait will be very promising for crop breeding.

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