Can we discover novel pathways using metabolomic analysis?

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Metabolomic analysis aims at the identification and quantitation of all metabolites in a given biological sample. Current data acquisition and network analysis strategies are classified on the basis of pathway elucidation and characteristics of theoretical networks. The development of metabolomic methods and tools is progressing rapidly, but an understanding of the resulting data is limited owing to a fundamental lack of biochemical and physiological knowledge about network organization in plants.

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Abbreviations

ECD	electrochemical detection
GC/MS	gas chromatography/mass spectrometry
IR	infrared spectroscopy
LC/MS	liquid chromatography/mass spectrometry
MS	mass spectrometry
NMR	nuclear magnetic resonance
RPLC	reverse-phase liquid chromatography
UV	ultraviolet

Introduction

Now that plant genomes have successfully been sequenced and (partially) annotated [1••,2], functional genomics has become a focal point for many research efforts. For *Arabidopsis* genes, over a third cannot be annotated by homology to genes in other organisms, and roughly 90% have not yet been experimentally investigated. Moreover, to see interactions between genes and gene products [3] (i.e. mRNA [4,5], proteins [6,7] and metabolites) and to look at their biological roles under different environmental situations, classical gene-by-gene approaches are not adequate. Much attention has been paid to transcript and protein profiling; however, for the rapid and statistically sound generation of interaction networks, these methods may be too slow and too expensive to be applied in large genomic studies [8•]. Furthermore, the underlying hierarchical paradigm of 'genes' as primary actors and 'gene products' as unwilling victims is questionable, because the regulation and control of metabolic fluxes may occur on all levels, as has been shown in a case study for the regulation of glycolysis [9••].

Despite extensive knowledge of fundamental metabolic processes, the mechanisms of physiological modulation over short and extended time intervals in response to changing environmental conditions remain difficult to understand [10••]. What is more, the pure existence of some plant metabolites such as trehalose [11] still puzzles us. Correspondingly, investigation of metabolic network regulation upon genetic or environmental perturbations may be viewed as a necessity for pathway discovery and functional genomics. There is a long tradition of, and extensive knowledge about, metabolite analysis. In fact, metabolite analysis can be better understood by distinguishing among levels on the basis of its objectives [12]. Four levels can be identified. First, there is metabolite target analysis, which utilizes specialized protocols for the analysis of difficult analytes such as phytohormones. Second, metabolite profiling aims at quantitation of several pre-defined targets (e.g. of all metabolites of a specific pathway or a set of metabolites typical for different pathways). Third, metabolomics has the ultimate goal of unbiased identification and quantitation of all the metabolites present in a certain biological sample from an organism grown under defined conditions. Fourth, there is metabolic fingerprinting, which, instead of separating individual metabolites by physical parameters, focuses on collecting and analyzing data from crude metabolite mixtures to rapidly classify samples. Among these four approaches, metabolomics seems to be best suited for investigation of metabolic networks, because it focuses on quantifying individual metabolites without having a bias concerning the choice of targets to be analyzed, as in metabolite profiling. Therefore, this review seeks to answer the question: are current strategies and methods of data acquisition and network computation sufficiently developed to adequately analyze and understand metabolomic networks?

Metabolomic data acquisition

The number of metabolites present in the plant kingdom is estimated to exceed 200 000. Therefore, metabolomic approaches must apply adequate tissue sampling, homogenization, extraction, storage, and sample preparation methods to maintain an unbiased process. Currently, no comprehensive comparisons of extraction techniques have been published that show high reproducibility, robustness, and recovery for all classes of compounds. For example, quite often, multiple components from homogenized tissues are extracted using alcohols or water/alcohol mixtures [13,14], but no systematic and rigorous validation [15••] has been published for extremes in plant tissues (such as *Arabidopsis* roots, strawberry fruits or pine needles). The same is true for other extraction techniques such as pressurized liquid extraction [16], supercritical fluid extraction [17–19], sonication [20], subcritical water extraction [21], microwave techniques [22] or pervapouration [23]. Additionally, it is quite unclear which factors most affect robustness, which is defined by minimal analytical errors if protocols are carried out under slightly altered conditions.

Such alterations may include subtle differences in extraction times, temperatures, solvent compositions and qualities, staff skills, tissue/solvent ratios, and others, with the potential to cause severe problems in reproducing results in different biological laboratories.

Numerous techniques exist for metabolite detection. It is questionable if data acquisition of a single physical parameter can fulfil the minimal requirements of metabolomic approaches, that is, comprehensiveness, selectivity, and sensitivity. Mass spectrometry (MS) seems to be the primary candidate to fulfil these criteria, as many papers have shown its suitability for metabolite detection in complex matrices [24••,25]. However, it is well known that gas chromatography/mass spectrometry (GC/MS), for example, is hardly applicable for organic diphosphates, cofactors or metabolites larger than trisaccharides. Electrospray or chemical ionization interfaces in liquid chromatography/mass spectrometry (LC/MS) result in bad ionization efficiencies for some metabolite classes such as carotenoids, hence limiting sensitivity and universality for metabolomic purposes. This lack of comprehensiveness is even worse if crude mixtures are not chromatographically separated before MS. The effects of ion suppression owing to matrix effects are well known to mass spectrometrists [26•,27], and these can be resolved only partially by the reduction in size of liquid droplets [28]. Apart from the problem of isomer distinction, such matrix effects invalidate any approach to large-scale pathway elucidation or metabolic reconstruction that fails to utilize chromatography or other means of physical pre-separation before metabolite detection. Instead, direct-infusion MS is ideally suited for the high-throughput classification of sample origins, as any matrix difference will have immediate and large effects in distinct mass spectra [29•,30] and ion abundances.

For the reasons given above, chromatography is therefore a prerequisite for pathway elucidation. Separation efficiency is roughly 10-fold better in capillary GC compared with regular LC columns. Therefore, more attention to chromatography must be paid in LC than in GC applications. Reverse-phase liquid chromatography (RPLC) of nonpolar organics is the classic and well-established means of separation before MS, but it regularly fails for ionic or highly polar metabolites. For analysis of oligosaccharides and sugar nucleotides in phloem exudates, LC/MS coupling has been achieved by hydrophilic interaction chromatography, resulting in better peak shapes compared with normal phase LC [31]. Hence, an LC-LC coupling of different chromatographic columns before metabolite detection seems to be a requirement for truly metabolomic approaches; however, no method has yet been developed that is as successful as coupling ion exchange to RPLC in the analysis of peptide mixtures [32••].

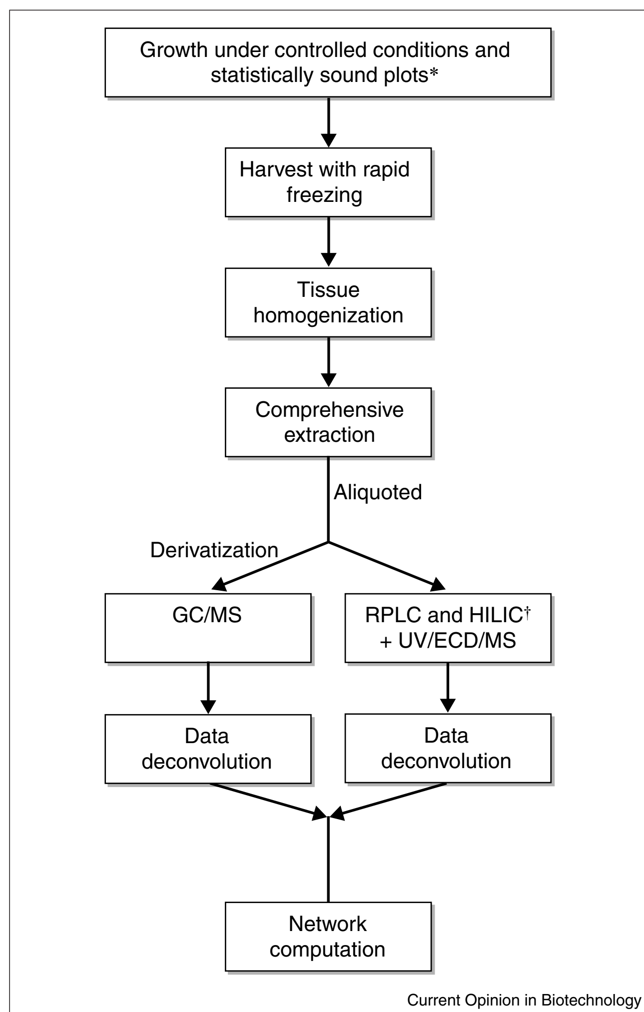
By extending this argument, it is clear that GC/MS and LC-LC/MS approaches have intrinsic biases against certain classes of compounds. Which other types of detection could lend a hand to MS? To this end, electrochemical detection (ECD), nuclear magnetic resonance (NMR), infrared (IR), ultraviolet (UV) or fluorescence spectroscopies may be applied. UV and fluorescence detection are well-known, non-destructive tools for metabolite target analysis or for profiling selected classes of compounds such as amines [33], isoprenoids [34] or unsaturated fatty acids [35]. Weaknesses of MS could further be complemented by applying coulometric electrochemical array detectors, which have been shown to be powerful and sensitive detectors of carotenoids [36], polyphenols [37], flavonoids, and others. Notably, this approach also enables the distinction of metabolite isomers [38] from spectral information, which is regularly hard to do by MS. Alternatively, IR and NMR spectroscopy might be considered; however, these approaches share the problem of lacking sensitivity for multiparallel analysis of hundreds of metabolites for generation of large metabolic networks. Nevertheless, NMR spectroscopy has high potential to unravel metabolic fluxes in branched, short pathways, if carried out together with isotope labeling and metabolic flux balancing calculations [39,40]. In addition, NMR spectroscopy [41] has been shown to have high discriminatory power on the level of metabolic fingerprints, for example, for the rapid assessment of the mode of action of plant protectants [42•].

If a combination of LC-LC and different detectors could potentially be so powerful, the parallel use of GC/MS might be questionable. Right now, however, GC/MS may still be viewed as the gold standard of metabolomic techniques due to the high separation power of GC and, more importantly, the better deconvolution algorithms [43•] that are available for classical quadrupole mass spectrometers or novel time-of-flight instruments [44]. Correspondingly, the parallel use of GC/MS and an LC-LC/UV/ECD/MS method could be expected to comprehensively cover plant metabolomes, allowing accurate identification and quantitation of plant metabolites for network computation (Figure 1).

Metabolic networks

Ideally, metabolomic data should accurately describe physiological processes as responses to developmental, genetic or environmental changes. However, some theoretical considerations limit direct interpretation of metabolic networks generated from metabolic snapshots. First, any subcellular compartmentalization is lost in the process of sample preparation. Although mRNA or protein expression levels can sometimes be ascribed to plant compartments on the basis of their target sequences, there is a high degree of uncertainty about the actual location of metabolites, many of which may occur simultaneously (and for potentially different purposes) in different locations and in varying amounts. Therefore, at best, metabolomic information can be interpreted on the multicellular, tissue or organ level. If metabolite analysis of subcellular compartments is the goal, large amounts of tissue must be used for the parallel determination of enzyme activities for ascribing cellular compartments to density fractions [45]. Because plant metabolomes are so complex, many, if not most, of the detected metabolites will remain structurally unidentified

Figure 1



Proposed scheme for comprehensive metabolomic data acquisition. *Plots need to be randomized according to the question under study (e.g. by latin square design). †HILIC (hydrophilic interaction chromatography) is a variant of normal phase chromatography, suitable especially for highly polar metabolites.

until being elucidated by *de novo* identification [46], which is much more difficult than the identification of transcripts or proteins. Finally, the question arises of how to correlate metabolite levels under different situations, if they only relate to multiple steady-states without any kinetic experimental design that could guide interpretation. Most often, average metabolite levels are used for deducing novel insights into plant physiology. This strategy again results in a loss of information, however, as metabolomic data from individual snapshots can be regarded to be as reliable as proven by the initial method validation tests. Any variation found in a homozygous plant population therefore indicates responses to subtle differences in plant development or physiology for each individual plant. This variation must have biological causes reflecting the flexibility of metabolic networks in the studied populations. It can, therefore, be used to calculate pathways by comprehensive pair-wise metabolite correlation plots. This idea was pursued by Arkin and colleagues [47] who demonstrated the deduction of pathways using only a few metabolites in a test case study of a kinetic experiment. Such correlations have also been observed in metabolite profiles from *Arabidopsis* leaves [48] and potato tubers [49], indicating that pathway discovery from a multiplicity of individual snapshots should be feasible despite a strong overlap of hundreds of simultaneous reactions and processes [50••]. Correspondingly, the observation of such fixed co-regulation of metabolite levels may force us to review the concept that metabolite ratios rather than metabolite levels are homeostatically regulated [51]. In the 1990s, the concept of 'metabolic control analysis' [52,53••] was extended by Hofmeyr [54] to incorporate metabolite co-response coefficients [54], which is essentially identical to metabolite:metabolite correlations if logarithmic data transformations are applied. If such correlation networks are visualized [48], differences to networks derived from other populations or static networks may be searched to generate novel hypotheses about biochemical pathways and gene functions. But, to which static networks could these metabolite co-regulation plots be compared? On the one hand, full genomes may be used to reconstruct what is metabolically feasible for each organism. On the other hand, however, these static networks are necessarily incomplete as genes may have more than one function, many genes do not show high homology to known enzymes, and homology itself does not always imply a coding for functionally related enzymes. Despite these constraints, stoichiometrically feasible metabolic networks could be computed for a variety of organisms. Such networks would enable researchers to predict the effect of knockout mutations [55••] and novel metabolic pathways [56••]. Besides allowing comparison with experimentally established metabolic networks, the inherent characteristics [57,58] of topological metabolic networks could be investigated to compare structural differences in network organization and thus improve our understanding of key metabolites [59] and the effects of random mutations [60]. Theoretical and experimental networks both reveal enormous complexity. This finding might entice researchers away from the dogma of 'fixed' metabolic pathways to a view that considers 'preferred' routes through biochemical networks [61•] as pathways that might be changed in response to altered conditions or needs. Such a view could lead to a better understanding of silent mutations or 'failed' antisense approaches for which no phenotypical change is observed even when the expression of important genes is down regulated.

Conclusions

Improvements in current metabolomic data acquisition technologies can be foreseen, especially for methods based on liquid chromatography. The first steps have been made to generate biological hypotheses from metabolomic datasets, however, such steps must be extended by better use of statistics to gain significant, rather than clustered, information. An understanding of metabolic networks might be further improved by an integration of static

enzyme stoichiometry networks and inherent network characteristics. Eventually, the combination of metabolomic analysis with other profiling technologies, especially proteomics and integrative techniques like metabolic control analysis [62], could enable pathway discovery and aid the evaluation of changes in plant networks engendered by genetic or environmental perturbation.

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