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Review

The future of metabolic phytochemistry: Larger numbers of metabolites, higher resolution, greater understanding

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Abstract

Like all biological disciplines, phytochemical research has seen profound changes in recent years. Whilst enzyme and metabolite purification and characterisation and pathway identification remain vastly important, the relative ease at which we can now obtain genome scale information has facilitated analysis at the level of the metabolic network. In addition, in recent years we have experienced an explosion in the number of plant proteins for which structural information is available. However, despite the presence of sequence information from a growing number of photosynthetic species, the function of many genes, let alone their in vivo roles, remains unclear. This review attempts to provide both an overview of the current state of the art and a perspective of the major challenges that remain.

Keywords: Metabolic profiling; Pathway analysis; Temporal resolution; Spatial resolution

Contents

1. Introduction

In this review, I shall present an assessment of the current state of the art in phytochemical analysis at the metabolite level, followed by a personal perspective of what I believe to represent the greatest challenges for the future. Several candidates immediately spring to mind when one attempts to define pivotal developments in phytochemistry, many of which are defined in Chris Somerville's excellent trajectory of 20th Century plant biology (Somerville, 2000). These include the vital elucidation of various aspects of photosynthesis (Kortschak et al., 1965; Calvin, 1962); the discovery of transposable elements (McClintock,

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1951); the establishment of the pathway of chlorophyll biosynthesis (Wolff and Price, 1957); the endosymbiont hypothesis of organelle acquisition (Gray et al., 1999); the high level structural resolution of photosystems I and II (Jordan et al., 2001; Zouni et al., 2001); the sequencing of the Arabidopsis genome (TAGI, 2000). Likewise key technological developments have underpinned these achievements, such as the generation of immobilised arrays for detecting multiple transcripts, proteins and metabolites and developments in sequencing, mass spectrometry and nuclear magnetic resonance spectroscopies, and computational analysis of metabolite networks. To these can be added several simple developments that we now take for granted, including the development of the automated pipette, tissue-culture expertise, the iterative improvement of molecular cloning techniques and ever-improving controlled-environment growth chambers. Such advances, both conceptual and practical, have allowed a broader and simultaneously deeper analysis of metabolism, than was possible a mere 10 years ago. Genetics has traditionally played an important role in the elucidation of plant metabolic pathways with Mendel's famous experiments on pea mutants, inadvertently studying a starch branching enzyme (Bhattacharyya et al., 1990). Many other early studies exploited mutants in combination with either steady state or isotope-labelled metabolite analyses (Calvin, 1962; Gage et al., 1997; Wheeler et al., 1998). This approach is currently still being followed successfully (Green and Fry, 2005; Kim et al., 2004; Masse et al., 2004; Schwender et al., 2004; Bao et al., 2002). However, the dependence of metabolic studies on genetic polymorphism has also considerably diversified to include both natural variation (El-Lithy et al., 2005; Schauer et al., 2005a; Kliebenstein et al., 2001; Bentsink et al., 2003; Sessa et al., 2000) and forward and reverse genetic approaches (Zhang et al., 2006; Schauer et al., 2006; Tieman et al., 2006; Coque et al., 2006; Fridman et al., 2004; Degen et al., 2004; Sergeeva et al., 2004). In parallel, integrated genomics approaches are beginning to yield an enhanced mechanistic understanding of biological systems – particularly when studied kinetically, following an environmental perturbation or spanning several developmental periods. These approaches are both discussed in detail in this review but, as technical aspects have been expertly reviewed elsewhere (Sumner et al., 2003; Kopka et al., 2004), I will concentrate here on physiological aspects. That said, the existence and maintenance of permanent genetic resources (see Gebhardt et al., 2005 for a recent review) places even greater importance on emergent databases (Zimmermann et al., 2004; Steinhauser et al., 2004), both for the storage and the effective evaluation of multi-level genomics data. Additionally, if such data are to be of use, certain quality criteria need to be respected. The metabolomics community is beginning to follow the lead set by transcript profilers to ensure that this is indeed the case (Jenkins et al., 2004; Bino et al., 2004; Fiehn et al., 2006). However, the stringency level of these criteria probably needs to be raised in these efforts to ensure a more robust documentation of how faithfully an extract represents the levels of metabolites present in vivo. In particular, experiments which investigate a novel tissue require robust validiation of the methods applied (Kopka et al., 2005; Lisec et al., 2006). One simple way of achieving this is to run "recombination experiments" in which any novel extract is run alongside and mixed with a previously characterised reference extract (Roessner-Tunali et al., 2003). This simple experiment allows the assessment of both the suitability of the extraction technique for the novel tissue and a validation of the qualitative peak assignments.

Beyond such issues, two great challenges remain to be solved in order that we can construct a higher resolution picture of metabolism. The first of these is to extend considerably coverage of the plant metabolome. The second is to understand better plant metabolism at the organ, tissue, cellular and sub-cellular levels. Since these both represent massive goals, much effort will be required before either is achieved. Before I discuss in detail current and potential approaches to tackle these challenges I would like to discuss, briefly, the benefits that could be gained from achieving them.

Within 50 years the problems of global hunger and environmental deterioration will both have considerably worsened (Morgan et al., 2006; Wolf et al., 2003) and, additionally, industrial growth will most likely have accelerated the depletion of biodiversity, thus robbing the world of many potential valuable plant-derived resources (Lopez-Pujol et al., 2006). The generation of plants displaying elevated rates of photosynthesis (Miyagawa et al., 2001; Lieman-Hurwitz et al., 2003; Pellny et al., 2004; Nunes-Nesi et al., 2005) and the development of biofuels and other uses of the under-exploited parts of plants grown for food use (Wolf et al., 2003; Morgan et al., 2006) are thus both important and necessary. Along with food production, healthcare issues are of crucial significance to people in underdeveloped regions (Yonekura-Sakakibara and Saito, 2006). Golden Rice, in which the ability to produce large amounts of β-carotene in cultivars of a familiar crop species will provide tangible benefits to meet social and economic needs by alleviating the human and social costs associated with blindness and other disabilities associated with vitamin A-deficiency, provides a good example to follow. It appears increasingly likely that the large scale generation of nutraceuticals and edible vaccines without requiring industrial facilities will become feasible (see Kumar et al., 2005; Ma et al., 2005 for examples). This area of research is currently rapidly expanding, as is the removal of antinutrients such as allergens (Lorenz et al., 2006). The increased coupling of plant- and nutrition-based research objectives (see Rein et al., 2006) should further aid in the development of the so-called functional foods. Moreover, the wide application of genomics tools to endogenous wild species will greatly aid efforts to "bioprospect" for pharmaceutically active compounds. Ultimately, human exploitation of bioactive metabolites will require their

identification and the identification of the regulatory properties of the metabolic pathways of their synthesis and degradation.

Whilst enhancing our completeness of metabolome analysis has clear applied aspects, high-resolution studies of the spatial basis are likely to have great utility in the definition of mechanistic understanding of the cellular (or organellar) response to circumstance. For this reason I will also detail both historical and contemporary methods aimed at improving our comprehension of spatial aspects of metabolism.

2. Metabolic diversity in plants

The genome of *Arabidopsis* contains approximately the same number of genes as that of the human. However, the comparison of the metabolite complement of these two species reveals a much greater metabolic diversity in the plant than the mammal (De Luca and St Pierre, 2000; Fernie et al., 2004; Hall et al., 2002; Fiehn, 2002). The most likely explanation for this is that such a complex metabolism has evolved in plants due to their lack of motility. This renders metabolic responses to stress and to interactions with its environment primordial features of a plant's metabolic arsenal (Pichersky et al., 2006; Goff and Klee, 2006; Kappers et al., 2005). To meet such demands it has been estimated that the plant kingdom contains upwards of 200,000 metabolites (De Luca and St Pierre, 2000) with values for a single species being given in the order of 15,000 (Dixon, 2001; Hartmann et al., 2005). A likely mechanism by which such huge diversity has come about is the combination of gene duplication and subsequent divergence of the resultant paralogs resulting in slightly diverse catalytic functionality (TAGI, 2000; Kliebenstein et al., 2001; Ober, 2005; Pichersky and Gang, 2000; Benderoth et al., 2006).

Current metabolite profiling methods cover a mere fraction of the metabolite complement of the cell, with GC–MS-based protocols only able to detect unambiguously 150 polar non-volatile small molecules (Roessner et al., 2000; Fiehn et al., 2000; Schauer et al., 2005b) or approximately 60 volatile compounds (Tikunov et al., 2005). Although the number of peaks is far in excess of this, they are not assigned and remain as "unknown" or "putative", unconfirmed by reference to authentic standards. The coverage afforded by a typical chromatogram from the polar phase of a sample is represented in Fig. 1, which shows this method appropriate for the study of central metabolism. The study of tomato volatiles mentioned above rendered

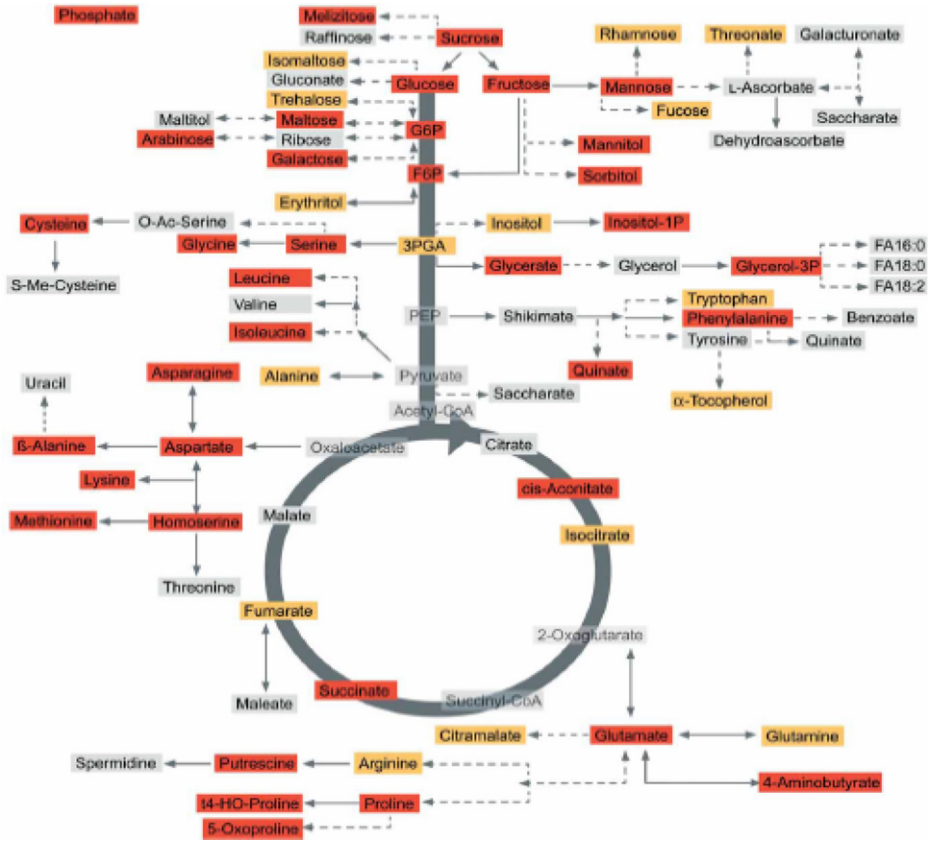

Fig 1. Pathway based overview of the level of coverage of primary metabolism afforded by current GC–MS based metabolic profiling approaches. The figure is an overview of experimental data, indicating metabolites that are associated and independent of metabolic change and is taken from Schauer et al. (2006).

at least putative indications of 80% of the volatiles that were previously reported to be present in this species (Petro-Turza, 1987). Similarly, LC–MS based methods are in existence for all major compound classes isolated from plants, including nitrogenous compounds, phenylpropanoids, benzoids, flavonoids, terpenes, cyanogenic glycosides and alkaloids.

Representatives of these classes present in the model plant *Arabidopsis thaliana* are shown in Fig. 2 (Narasimhan et al., 2003; Hagemeier et al., 2000; Rohde et al., 2004; Hemm et al., 2004; Debeaujon et al., 2003; Chen et al., 2003b; Tattersall et al., 2001; Wu et al., 2006; Bringmann et al., 1999). However, attempts to establish a profiling method that allows unambiguous detection and determination across the spectrum of compound class have thus far only allowed the development of a method capable of unambiguously determining a few metabolites, even though the detection of some 2000 mass peaks is possible (Von Roepenack-Lahaye et al., 2004). The recent publication of a database for the secondary metabolites of tomato (Moco et al., 2006) represents a promising step towards expanding this list, and peak identification methods have dramatically improved the ability to determine the proportion of "putative" metabolites (Keurentjes et al., 2006). A shift towards confirmed metabolite identity is undoubtedly the next necessary step, but this is somewhat hampered by the complex chemistry behind the synthesis of many secondary metabolites and a consequent shortfall in commercially available products for this class of metabolites. The other approach is the continued development and improvement of highly specific methods for the analysis of defined classes of chemicals (see for example Tolstikov et al., 2003; Farag et al., 2006; Li et al., 2006; Garratt et al., 2005; Chiwocha et al., 2003; Weichert et al., 1999). Such approaches have been commonly taken for the secondary metabolites mentioned above, as well as for photosynthetic pigments (Enfissi et al., 2005) and cell wall components (Lerouxel et al., 2002). However, although currently limited, the number of metabolite levels that can be determined by MS-based methods is far in excess of what can be measured by NMR spectroscopy, which is applicable only to a small number of abundant metabolites. NMR does, however,

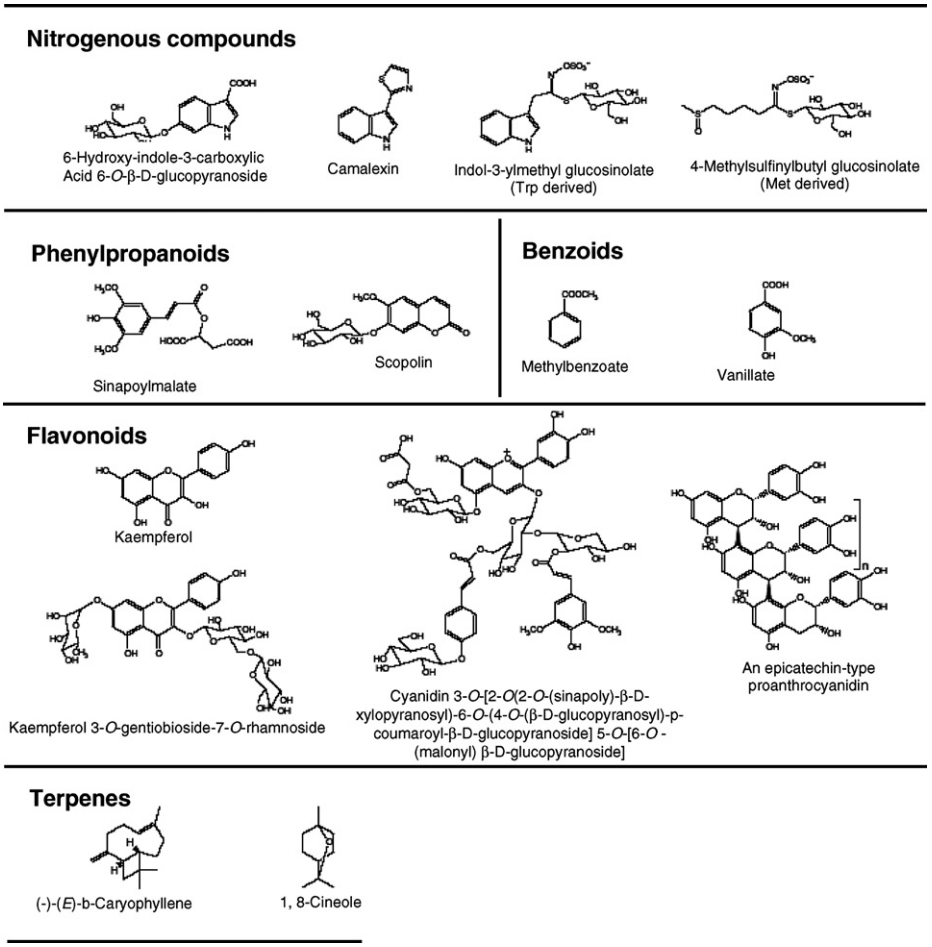

Fig. 2. Examples of plant secondary metabolites arranged by major class. Adapted with permission from ÓAuria and Gershenzon (2005). Selected representative metabolites are presented. The family in which they belong is indicated in the figure. Further subdivision is also possible for the following compounds (subfamily given in parenthesis): camalexin (an indole-sulfur compound), scopolin (a coumarin), kaempferol 3-*O*-gentiobioside-7-*O*-rhamnoside (a flavonol glycoside), cyanidin 3-*O*-[2-*O*(2-*O*-(sinapoly)-β-D-xylopyranosyl)-6-*O*-(4-*O*-(β-D-glucopyranosyl)-p-coumaroyl-β-D-glucopyranoside] 5-*O*-[6-*O* -(malonyl) β-D-glucopyranoside] (an anthrocyanin), (−)-(*E*)-β-caryophyllene (a sesquiterpene) and 1,8-cineole (a terpene).

display the advantage over MS-based techniques in quantifying fluxes and also to some extent in studies on the compartmentation of metabolism (Ratcliffe and Shachar-Hill, 2006). Despite recent advances in the sensitivity of NMR spectroscopy, it would appear likely that coupled MS technologies will continue to represent the tool of choice for broad-range metabolite analysis. The MS methods mentioned above all rely on standard quadrupole or time-of-flight separation. Another approach worthy of note is fourier-transform ion cyclotron resonance mass-spectrometry (Oikawa et al., 2006; Breitling et al., 2006; Aharoni et al., 2002). This technique affords unparalleled mass accuracy but currently suffers from two pitfalls. Perhaps the more trivial of the two is that, when used in isolation, it is unable to differentiate isomers. However, this could be overcome either by running a chromatography step to allow prior separation or by running multidimensional MS in order to fractionate metabolites into signature fractions specific to a certain isomer. A less trivial problem is that the documentation given thus far to support broad (Aharoni et al., 2002), as opposed to focussed (Oikawa et al., 2006; Schmidt et al., 2007), metabolite profiling is insufficient to assess the quality of this approach. It would seem likely that the increasing efforts to enforce reporting standards for metabolomics may lead to improved certification of its credentials for this purpose.

As for the metabolites themselves, identification of the genetic basis of metabolite diversity has also seen dramatic progress in the last decade. This includes analysis at the level of gene functional annotation and gene family analysis (see Section 5), analysis of genetic factors underlying metabolite accumulation and metabolic regulation, studies on the heredity of metabolism and structure/function studies of genetic polymorphisms. These are all discussed in detail below. Likewise, at the protein level striking advances are also being made. Several different approaches are currently being taken in parallel. These range from the cataloguing of proteins (Wienkoop and Weckwerth, 2006; Taylor et al., 2005; Rossignol et al., 2006; Rose et al., 2004) and their post-translational modifications using proteomic techniques and robotized assays (Balmer et al., 2004; Nuhse et al., 2003; Gibon et al., 2004; 2006), through description of their kinetic parameters (Yang et al., 2005; Fridman et al., 2004), to their structural resolution (see for example Fieulaine et al., 2005). Through centrifugation-based approaches (Kleffmann et al., 2004; Sweetlove et al., 2002), as well as marker-based (Millar et al., 2006), molecular biological (Yamane et al., 2005) and computational approaches (Small et al., 2004), a relatively comprehensive picture of the compartmentalised localisation of proteins is being drawn up. Once better estimates of subcellular metabolite contents and kinetic parameters of the various isoforms of enzymes have been determined, our understanding of metabolic regulation at the network level will be far more complete. That said, this is no facile task since analysis of the *Arabidopsis* genome alone reveals it to contain in excess of 250 cytochrome P450 genes, 100 acyl transferase genes, 300 glycosyl transferase genes, 300 glycoside hydrolase genes and a large number of genes encoding enzymes such as dioxygenases, *O*-methyltransferases, terpene synthases, or polyketide synthases (http://www.Arabidopsis.org/info/genefamily/genefamily.html; Von Roepenack-Lahaye et al., 2004).

3. Advantages and limitations of working at the level of the metabolite

Despite the advances listed above in plant protein biology the focus of this article is on plant metabolites. Before discussing recent progress in this field it is pertinent to reflect on the advantages and limitations inherent in the study of metabolites. Firstly, the advantages. Metabolite measurements have been carried out for decades because of the fundamental importance of metabolites as components of biochemical pathways, the importance of certain metabolites in the human diet, and their use as biomarkers for a wide range of biological circumstances (Fernie et al., 2004; Hall, 2006). Moreover, systemic evaluation of all the metabolites within a given section of the metabolic network can facilitate the elucidation of key regulatory sites of metabolism (Stitt and Fernie, 2003), with the sites of regulation identifiable since the substrate(s) of the key enzyme(s) change reciprocally to the flux through the pathway (Rolleston, 1972). The theory can perhaps be best illustrated by taking as example nitrate assimilation. It is commonly observed that glutamine levels often rise or fall but that those of 2-oxoglutarate and glutamate remain unaltered (Scheible et al., 2000; Urbanczyk-Wochniak and Fernie, 2005), indicating that restriction of the flow of reduced nitrogen is regulated downstream of glutamine, thus pinpointing the enzymes GOGAT and/or glutamate dehydrogenase as targets for detailed studies to attain mechanistic insight (Stitt and Fernie, 2003). However, such a simple analyses is only valid for simple linear pathways or in instances in which such pathways dominate fluxes and therefore may have limited utility in the analysis of secondary metabolism. It has, however, been successfully applied to the sucrose-starch transition in the potato tuber (Geigenberger et al., 2004; Tiessen et al., 2002) and, once pathway saturation with regard to both metabolite measurement and genetic perturbation of other pathways is achieved, is likely to be a highly informative strategy to analyse metabolic regulation of primary metabolism. The vast improvement in our ability to document changes in metabolite contents from (previously) unconnected pathways is also facilitating a better understanding of how the cell prioritises partitioning of important nutrients in a wide range of conditions. Many recent studies have implicated metabolites in the mediation of gene expression (Lancien and Roberts, 2006; Baier et al., 2004; Laule et al., 2003; Fujiki et al., 2001; Sheen, 1994) and as key players in determining responses to perceived stress and developmental cues (see for example Panicot et al., 2002; Palanivelu

et al., 2003). Such demonstrations that metabolite levels influence gene expression, alongside suggestions (largely from studies in yeast) that they can additionally influence protein stability (Singer and Lindquist, 1998; Fafournoux et al., 2000) and act as mobile cellular and intracellular signals are counter to the of-made suggestion that metabolites are the final recipients of genetic information. There is, however, little doubt that the phenotype of any biological system is largely determined by its metabolite composition, giving a strong reason to invest research effort into furthering our understanding of how this is mediated. The fact that metabolomics studies entities that operate at a level directly relevant to biological function, is perhaps the key reason for their study. In summary, metabolites are highly regulated, integrated and integrating components of the cell, with roles ranging from energy and redox control to defence and resistance and from structural integrity to signalling, and their study yields direct insight into the points of cellular control both of metabolism per se and the many other biological processes with which it is intimately associated.

Set against these advantages is the undeniable fact that the measurement of metabolites is difficult. This is principally due to their dynamic behaviour and the diversity of their chemical nature (Stitt and Fernie, 2003). Many metabolites change during transition between conditions and even within steady state conditions, with half times of hours, minutes, seconds and even fractions of seconds (Lilley et al., 1982; Stitt and Gross, 1988; Scheible et al., 2000; Kleiner et al., 1999; Kruse et al., 2003). Historically, this has been problematic since it required the development of techniques to quench metabolism rapidly (techniques that subsequently facilitated the determination of the subcellular distribution of metabolites). Also, since the low concentrations of many metabolites make the sampling of large tissue amounts a pre-requisite, the number of experiments analysing the kinetics of metabolite accumulation (or degradation) has been relatively limited. Moreover, the presence and regulation of large numbers of specific and non-specific transporters at both the organellar and plasma membranes dictates that many metabolites are present in multiple compartments (discussed in more detail below). A further historical problem was the fact that metabolites were generally measured individually using spectrophotometric methods or in mixtures of low complexity using chromatographic methods. Such methods were relatively easily optimised, such that extraction buffers used could be customised for all but the most labile of metabolites. The chemical diversity of metabolites is much greater than that of nucleic acids and proteins due to the enormous diversity in there structure. As would be expected this structural diversity confers a broad range of chemical properties which produce the problem of finding a broadly suitable extraction buffer. At present, even the combination of all the protocols listed above (spanning a wide range of analytical tools) allows us to see only a small fraction of the total metabolome. Although one problem is simply that of scale with several thousand metabolites present in any given plant species, this is not the only problem (Fernie et al., 2004). The dynamic range of concentrations of metabolites is also huge. Why this is so can partially be explained by the variation in size between small metabolites such as C1 compounds or co-factors such as metal ions up to the giant macromolecular structures of starch and lignin. However, there are several other reasons for maintaining the concentrations of some metabolites at low levels, including their toxicity, the propensity of certain functional groups to undergo non enzyme catalysed transformations, energy considerations and the simple constraint that there is not enough room in the cell for every metabolite to be present above trace levels (Fell, 1997; Sweetlove and Fernie, 2005). Although there are clearly important reasons underlying the dynamic range of metabolite level, the upshot is that it causes problems for the experimentalist since few analytical platforms can cope with such an extensive range (see Sumner et al., 2003 for details). In some instances there are trivial ways around the problem, such as concentrating or diluting extracts extracts (such as those required for assessing tomato fruit development, Roessner-Tunali et al., 2003; Carrari et al., 2006). An alternative is the isotopic labelling of metabolites to enhance sensitivity (Birkemeyer et al., 2005; Engelsberger et al., 2006). A greater problem than the diversity in dynamic range is, however, that apparent in their chemical functionality. This diversity means that there is no single extraction process that does not incur substantial loss to some of the cellular metabolites, let alone a single analytic platform that can measure all metabolites. A second problem that has been exacerbated by the use of mass spectrometry, is the number of analytes of unknown chemical structure. Typically in current GC–MS based metabolite profiling a chemical structure has been unambiguously assigned in only 20–30% of the analytes detected (Roessner-Tunali et al., 2003). The solution to neither problem is trivial and will most likely require successive iterative improvements. Sample pre-processing and solid phase extraction (see for example Tolstikov and Fiehn, 2002; Tolstikov et al., 2003), and a fuller integration of the chemistry-specific measurement platforms could improve coverage. In the longer term, new technologies will likely ameliorate the problem. Obvious candidate technologies include chromatographically coupled FT-ICR-MS and improvements in multidimensional MS and NMR as well as the coupling of the two is likely to substantially improve coverage. These developments clearly need to go hand-in-hand with the establishment of web-based databases for the academic community.

4. Systems responses to changes in genotype, prevailing environmental conditions, biotic and abiotic stress

The last five years have witnessed a genomics-driven renaissance in holistic analysis, or at least in holistic thinking (for example see Bothwell, 2006; Trewavas, 2006;

Sweetlove and Fernie, 2005; Thum et al., 2003; Raikhel and Coruzzi, 2003). Whilst many disciplines of plant biology have embraced such approaches for some years, the emergence and adoption of the transgenic approach with its focus of directly understanding the function of a given protein generally led to a more reductionist outlook. Whilst transgenic studies undoubtedly increased our understanding of plant metabolism enormously, researchers have often been confronted with unexpected changes. For example, very many biotechnological attempts to produce increased starch accumulation in the potato tuber gave the opposite result (Fernie et al., 2002). In order to understand the reasoning behind these unexpected results, a broad analysis of one such example - that of invertase-overexpressing tubers – was carried out at the level of transcript, enzyme activity, metabolite levels and fluxes (Urbanczyk-Wochniak et al., 2003; Sonnewald et al., 1997; Trethewey et al., 1998; Roessner-Tunali et al., 2004; Bologa et al., 2003). From this large cumulative dataset it was possible to conclude, that the lines were characterised by an upregulation of carbohydrate oxidation, that was most probably caused by an increased demand for ATP imposed on the cell by a large increase in the cycling of sucrose due to similtaneously high rates of its synthesis and degradation. At the phytochemical level such approaches are now commonly adopted most visibly at the level of the transcriptome, proteome and metabolome (see for example, Locke et al., 2007; Dodd et al., 2005; Heazlewood et al., 2004; Carter et al., 2004; Goossens et al., 2003: Chen et al., 2003b). Early studies at the later two levels were essentially based largely on qualitative documentations of presence/absence in various tissue types or following genetic manipulation.

More recently, however, studies are getting increasingly sophisticated. As the state of the art of the use of metabolic profiling (on its own and in conjuncture with other genomic tools) has recently been extensively reviewed (Schauer and Fernie, 2006) and there are a number of other recent reviews on integrated genomics (Aoki et al., 2007; Oksman-Caldentey and Saito, 2005; Lange, 2005; Fridman et al., 2005) I will here just highlight major findings and future areas of development of this area of molecular physiology. Genomic-scale analytic tools currently allow us far greater ability to monitor the cellular response to a perturbation of circumstance than was available even ten years ago. Their development was paralled by a dramatic increase in our ability to generate and genotype diverse genetic populations (see Roessner et al., 2001 for details) as well as increasing awareness of factors involved in perception and response to biotic and abiotic stress (Bohnert et al., 2006). When taken together, the upshot of these recent advances is the ability to describe cellular responses to various biological situations at a high level of detail. Two major types of information can be obtained from such studies. Firstly, they allow the identification of co-responses amongst molecular entities. For example, novel metabolic changes in response to cold stress and phosphate, nitrate and sulphur stress have recently been documented (Cook et al., 2004; Nikiforova et al., 2003; Hirai et al., 2004; Osuna et al., 2007), and a broader insight into the effect of altering redox status has been achieved (Balmer et al., 2003; Kolbe et al., 2006), adding further insight into processes that have been relatively well characterised for decades. Secondly, it can allow inference of mechanistic insights underlying the changes observed. Several examples of this are now apparent in the literature, particularly in instances studying the kinetics of the metabolic response. In the case of environmental perturbations, this can be performed relatively trivially by means of serial sampling of plant materials. The study of genetic perturbations requires the carefully controlled use of inducible promoter systems. Both approaches have, however, been tackled. Analysis of metabolic change at a broad level has been performed in diurnal experiments in potato (Urbanczyk-Wochniak et al., 2005) and *Arabidopsis* leaves (Bläsing et al., 2005; Gibon et al., 2006; 2004; Thimm et al., 2004). The experiments in potato revealed that few metabolites appeared to be under tight transcriptional control. A similar result was also found for *Arabidopsis*, in which the authors also measured a range of enzyme activities and were able to conclude that changes of transcript levels typically led to strongly damped changes of enzyme activity, suggesting that enzyme activities provide a quasi-stable integration of regulation at several levels and provide useful data for the characterization and diagnosis of different physiological states. Following a similar strategy, we were able to evaluate gene-metabolite networks underlying tomato fruit development (Carrari et al., 2006), revealing the metabolism of particular organic acids, of cell wall monomers and of pigments to be under tight regulatory control – most probably by a minor number of transcription factors and signalling components. Comparable yet distinct metabolic programming was detected following a similar approach to analyse the entire process of seed development in *Arabidopsis*. Other studies of this type include following dark-induced senescence (Buchanan-Wollaston et al., 2003; Ishizaki et al., 2005) and the progressive response to methyl jasmonate elicitation (Suzuki et al., 2005). In parallel, the creation of a range of inducible gene expression systems (Moore et al., 2006) has allowed genetic perturbation to be studied in a similar manner. This allows the dissection of primary and pleiotropic effects and has already proven useful in the example of the overexpression of invertase mentioned above (Junker et al., 2004).

As an alternative to measuring steady state is the analysis of metabolic flux. Tools for this purpose and suitable mathematical frameworks for their analysis are getting increasingly sophisticated (see Ratcliffe and Shachar-Hill, 2006 for details), and complicated flux models are beginning to appear based on both experimental (Baxter et al., 2007, in press; Schwender et al., 2004; Rontein et al., 2002) and theoretical (Steuer et al., 2006) information. Inherent in these anaylsis is a reliance on computer modelling. Mathematical approaches to understanding metabolic

regulation have long been applied to plants. The combination of genome sequencing and the construction of large-scale species-specific metabolic pathway maps (Zhang et al., 2005) have allowed this to be taken to a high level. Given that the various chemical entities of the cell respond at different rates to system perturbations (see Gibon et al., 2004), the utility of computer-based approaches in order to maximize our understanding of biological processes will likely expand dramatically in the coming years.

5. Gene annotation through genomics

Since the widespread adoption of transgenic manipulation of plants, much research focus has been centered on gene annotation. We are currently in the fortunate position that we have readily available tools for both forward and reverse genetics. Forward genetic screens have brought about great advances in our understanding of many metabolic pathways including, but by no means limited to, those involved in sugar (Huijser et al., 2000), starch (Smith et al., 2003), cell wall (Reiter et al., 1997; Zablackis et al., 1996), lipid (Arondel et al., 1992; Somerville and Browse, 1991), pigments (Isaacson et al., 2002; Niyogi, 1999) intermediary metabolism (see for example Feller and Fischer, 1994) and secondary metabolism (see for example Lehfeldt et al., 2000; Watson et al., 1998). However, despite the fact that genome sequence information is now available for a growing number of photosynthetic species (TAGI, 2000; Goff et al., 2002; Nakamura et al., 2002; Armbrust et al., 2004; Derelle et al., 2006), knowledge of gene function remains incomplete. To give but two examples, despite its prominence in glycolysis, information concerning the genetic identity of plant phosphofructokinase was only published this year (Winkler et al., 2007), and our genetic understanding of the entire ascorbate biosynthetic pathway still remains fragmentary (Conklin et al., 2006). Given our relative paucity of understanding of gene function, it is clear that the vast majority of phytochemical research in the next years will remain directed to this task.

Perhaps the simplest application of genomics to this problem is to study co-expression of genes using the ''guilt by association approach''. The principal here is that genes showing similar responses will perform similar functions. The establishment of publicly accessible microarray data repositories (Zimmermann et al., 2004; Steinhauser et al., 2004) greatly facilitated this approach and has helped to lead to the identification of many novel metabolism-associated genes including those linked to cell wall (Usadel et al., 2005), brassinosteroid (Rautengarten et al., 2005), glucosinolate (Hirai et al., 2007) and anthocyanin metabolism (Vanderauwera et al., 2005; Aoki et al., 2007; Yonekura-Sakakibara et al., 2007),. Furthermore, this approach has led to the classification of process-associated genes, including those linked to cold stress (Hannah et al., 2005) and jasmonate signalling (McGrath et al., 2005). Moreover, integration of data from various genomic strategies has also been recently demonstrated to be a highly effective means of gene function identification (see Fig. 3A). Most prominently, the use of metabolomics in conjuncture with knock-out mutagenesis in *Arabidopsis* has allowed the identification of the specific function of the PAL1 and PAL2 genes of phenylpropanoid metabolism (Rohde et al., 2004), the-myb like transcription factor PAP1 (Tohge et al., 2005) and cytochrome P450 CYP710A (Morikawa et al., 2006). Given that so many knockout mutants already exist in *Arabidopsis* and rice (Alonso et al., 2003; Hirochika et al., 2004), it is highly likely that this approach will greatly aid in gene identification in these species, at least in instances where the gene function is intimately associated with metabolites that we can readily and reliably detect.

In other plants species – that currently lack such genetic resources – targeted metabolite analysis in conjuncture with transcript analysis has allowed the elucidation of several genes involved in volatile synthesis (see for example: Fridman et al., 2005; Orlova et al., 2006; Schuurink et al., 2006; Schnee et al., 2006; Simkin et al., 2004), and secondary metabolism in general (see for example: Goossens et al., 2003; Chen et al., 2003b), whilst co-regulation between metabolites has also been used to infer pathway structure (Tikunov et al., 2005; Keurentjes et al., 2006; Ziegler et al., 2006; Zulak et al., 2007; Broun, 2005) and even metabolic regulation (Roessner et al., 2001; Fernie, 2003). Although finding correlative behaviour between these molecular entities gives an important clue to their function a biochemical demonstration of activity is necessary to establish proof, as pointed out by Pichersky and Niyogi (2006). The most common strategy is the heterologous expression of recombinant proteins and assaying of

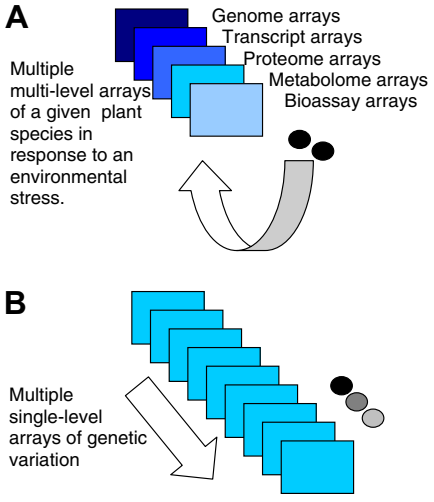

Fig. 3. Gene-functional annotation by array based methods. Panel A represents the multi-level approach wherein a few experimentally manipulated samples are characterised using multiple genomics platforms (this panel is modified from Oksman-Caldentey and Saito, 2005). Panel B represents the single level approach wherein many experimental samples are phenotyped using a single genomics platform (in this case metabolomics). Both approaches are currently finding wide utility in gene functional annotation studies.

enzymatic activities. Examples of this approach are legion, recently including characterisation of enzymes involved in carotenoid cleavage (Ibdah et al., 2006), the synthesis of phenylpropanoid and phenylalanine-derived volatiles (Guterman et al., 2006; Tieman et al., 2006; Kaminaga et al., 2006), and tocopherol (Porfirova et al., 2002).

The utility of informatics-based approaches is, however, by no means limited to correlation analysis with modelling approaches based on either dynamic (McNeil et al., 2000; Roessner-Tunali et al., 2004) or steady state (Schwender et al., 2003; Rontein et al., 2002) modelling following isotope labelling being increasingly readopted in order to better understand pathway and network function. An important recent example of this is the work of Schwender and co-workers which documented an important role of Rubisco outside of the context of the Calvin cycle in the maintenance of an efficient pathway of oil biosynthesis in the oil seed *Brassica napus* (Schwender et al., 2004). This approach utilized GC–MS based metabolite determinations in conjuncture with mathematical modelling using Schuster's elementary modes (Schuster et al., 2000) in order to elucidate the role and positioning of Rubisco in the metabolism of this tissue. As mentioned above, there is increasing evidence of enzymes playing more than one role in the cell. Whilst this is well documented in secondary metabolism, it is equally the case in primary metabolism. Therefore, even if the function of a gene is determined following heterologous expression, the testing of its function in planta using the reverse genetic approach will likely retain an important role to play in gene functional annotation for some years to come.

As an alternative route to gene identification the Quantitative Trait Loci (QTL) approach reveals genomic regions underlying variance in given phenotypes (represented in Fig. 3B). This approach has recently been taken to determine QTL for metabolite content and metabolic regulation of a broad range of primary metabolites in tomato (Schauer et al., 2006; Fridman et al., 2004), as well as for given compound classes of primary and secondary metabolites in *Arabidopsis* (Fernie et al., 2006; Morreel et al., 2006; Calenge et al., 2006; Liu et al., 2003). Similar approaches are currently being assessed at the level of enzyme activities (Cross et al., 2006; Obara et al., 2001; Thevenot et al., 2005). In addition, as a first step towards a broader profiling of secondary metabolism Keurentjes et al. (2006) recently mapped QTL for LC–MS derived mass spectral traces in *Arabidopsis*. Similarly, gluconsinolate profling alongside transcriptomic analysis and map-based cloning has facilitated the cloning of the Epithiospecifier Modifier 1 (ESM1). Subsequent in plant and in vitro analysis revealed that the gene underlying this QTL was a myrosinase-associated protein and that it repressed nitrile formation and favoured isothiocyanate production (Zhang et al., 2006). Although the QTL approach is currently widely adopted, these examples represent the first in which a broad survey of metabolism was carried out. The fact that metabolite profiling is largely species independent (Stitt and Fernie, 2003), suggests that this approach can be readily taken in many crop species in addition to *Arabidopsis*. However, it is worth noting that the time taken for fine-mapping QTL, even in species such as tomato for which high resolution maps exist (Mueller et al., 2005), is far in excess of that in *Arabidopsis*. For this reason alone it would appear likely that future approaches will concentrate on both the model species and economically important crop species in parallel. We are currently investigating the influence of gene/environment interaction on primary metabolic pathways in tomato fruit, as well as investigating the mode of inheritance of these traits. In addition, since the introgression line population represents a fantastic resource to study metabolic regulation of individual pathways and/or metabolites, we are attempting to gain a better understanding of organic acid (Nunes-Nesi et al., 2005) and branched-chain amino acid (Ishizaki et al., 2005, 2006) metabolism. Given that these examples include crop species, it holds promise that a combination of phytochemical analysis and marker-assisted breeding may provide an alternative strategy to transgenesis for metabolic engineering (Giovannoni, 2006; Dixon, 2005; Dixon et al., 2006).

Interestingly, the variation in metabolite content observed following the use of natural variance is similar in scope to that measured in transgenic approaches deemed as successful (Davuluri et al., 2005; Geigenberger et al., 2005; Regierer et al., 2002; Memelink, 2005; Van Camp, 2005; Fernie et al., 2006). Moreover, genetic populations such as those described above are proving of sufficiently high resolution to map traits down to the level of the gene or even a single nucleotide polymorphism within a gene (Fridman et al., 2004; Maloof et al., 2001; El-Assal et al., 2001). With this in mind, comprehensive metabolite profiling of wide genetic diversity will likely prove important in crop improvement with the mechanism behind the crop QTL of agronomic relevance already being determined (Doebley et al., 2006; Ashikari et al., 2005; Vigouroux et al., 2002). Given the insight obtained in these few studies, it follows that understanding of the genetic basis of crop quality alongside that of natural product synthesis will represent an important research frontier in future years. Important basic studies on the inheritance of agronomic traits (see Semel et al., 2006) and the ability to pyramid beneficial traits (see Gur and Zamir, 2004) will be fundamental to improving agronomic efficiency.

6. Spatial dynamics: understanding the interdependence of cells types and cells of the same type

Generally metabolism is studied in a tissue specific manner but cells within a given tissue are treated as homogeneous. This is clearly an oversimplication since there are around 40 different cell types in plants (Goldberg, 1988; Martin et al., 2001), with many of these having specialised function and metabolism. The majority of measurements are however made on a mixture of cell types and therefore

values presented generally better reflect the predominant cell type of a sample than the more specialised cell types. Examples of spatial dissection of metabolism are now abundant in the literature with several techniques being utilized over many years (Kehr, 2003; Borisjuk et al., 2002; Fehr et al., 2002; Liu et al., 1990). Technical innovation has, however, increased this resolution several-fold and now examination of gene expression and its consequence on metabolism in distinct tissues is now routinely carried out (see Joshi et al., 2007; Wagner et al., 2006; Schmidt et al., 2007; Delledonne et al., 1998; Dixon and Lamb, 1990; Kühn et al., 1997; Fischer et al., 1995; Sonnewald and Stitt, 1995; Pena Cortes et al., 1995 for examples). This is a particularly important development given that the size of gene families in plants dictates that family members often exhibit differential expression patterns and diversity in function. Although relatively trivial analysis of metabolism in different organs of the same plant is rarely carried out, in the instances where this has been attempted, the influence of specific genes or experimental conditions has often been found to be widely different (see Roessner-Tunali et al., 2003; Carrari et al., 2005; Desbrosses et al., 2005). First steps towards enhancing spatial resolution should therefore be aimed at this level. At least in *Arabidopsis,* the framework for these analyses is well established. Large insertion-mutant populations exist, in which individuals lack expression of a defined gene and the expression pattern is well catalogued both in the publicly-searchable microarray results (Zimmermann et al., 2004) and by the widescale expression of GUS-fusion proteins (Sundaresan et al., 1995). Moreover, the development of tissue-specific and chemically-inducible promoters allow the tightly controlled expression of transgenes within certain parts of the plant (Maizel and Weigel, 2004; Schaarschmidt et al., 2004; Junker et al., 2003; Ellerstrom et al., 1996; Müller-Röber et al., 1995; Leidreiter et al., 1995), circumventing the need for performing experiments on isolated organs or tissues. This is a particularly important advance, since the investigation of isolated cells such as protoplasts is problematic because plant cells quickly loose their specific gene-expression pattern when they are removed from their location within the tissue (Grosset et al., 1990). Moreover, destructive tissue-isolation protocols may cause wounding effects and may disconnect the selected tissue from supracellular signal transduction chains. Consequently, the results obtained from isolated plant cells may not reflect the *in vivo* situation (Kehr, 2001).

An even greater advance in this respect is the technological improvements that allow microdissection of tissues using so called single-cell techniques (Xiao et al., 2004; Chuong et al., 2006; Verscht et al., 2006; Nelson et al., 2006; Lange, 2005; Dharmadhikari et al., 2006). These allow more complete extraction than the minimally invasive extraction of cell contents with glass microcapillaries, which can be used alone or attached to a pressure probe (Kehr et al., 1999). After impalement, the cell's turgor pressure forces a portion of mainly vacuolar cell sap into the capillary. This technique can be modified to extract more cytoplasmic cell contents, for the investigation of macromolecules such as nucleic acids or proteins, by applying a negative pressure to the capillary (Brandt et al., 1999). Non-ambiguous cell identification can be achieved using fluorescent dyes to distinguish certain tissues or by coupling fluorescent markers to tissue-specific promoters. A recent advanced sampling method that circumvents the problem of limited starting material allows the precise collection of large homogeneous cell populations on the basis of direct microscopic visualisation. Laser-capture microdissection (LCM) can be applied to solid sections of fixed tissue and is used for the analysis of animal tissues (Emmert-Buck et al., 1996), especially in the clinical sector. The applicability of LCM to plant tissue samples is gaining pace (Cai and Lashbrook, 2006; Ramsay et al., 2006; Day et al., 2005; Schad et al., 2005; Casson et al., 2005) and this sampling method has the potential to open new possibilities for the high-resolution analysis of plants.

Highly-exciting exploitation of such single-cell methodology has already begun with cell specific transcript accumulation being catalogued as well as proteomic analyses and even metabolite analysis being carried out. At the physiological level, parallel recordings of photosynthetic electron transport and K^+ channel activity have been achieved in single guard cells (Goh et al., 2002). Methods have also recently developed for electroanalysis of metabolic fluxes in simple microsystems (Yasukawa et al., 2002). It would seem likely that further improvements will allow us to gain a much better cellular perspective of the metabolic basis of plant development and response to stress.

Going to even higher levels of resolution allows the analysis of sub-cellular and even sub-organellar aspects of metabolism. Generally, the spatial distribution of metabolites fits well to that of proteins involved in their metabolism, with organelle-specific isoforms or dual-targetting largely accounting for the duplication of metabolic reactions in the plant cell (Millar et al., 2006; Carrari et al., 2003). That said, there are several observations in which either enzymes or metabolites have been found in a subcellular location in isolation of one another. For enzymes, reasonable evidence exists that the nuclear location of hexokinase is due to a signalling, rather than a metabolic, function (Cho et al., 2006), and a similar role has been postulated for the recently-observed mitochondrial localisation of sucrose synthase (Subbaiah et al., 2006). The observation of enzymes in unusual locations is not confined to plants and numerous examples of secondary so-called moonlighting functions exist in diverse biological species (Jeffery, 2004; Moore, 2004; Matarasso et al., 2005). These are particularly well characterised within mammalian and microbial systems but, although they are largely uncharacterised in plants, the analysis of such functions is likely to become crucial in coming years. Another recent example of this is the localisation of a complete glycolytic pathway functionally associated with the mitochondria in

Arabidopsis (Giege et al., 2003). However, given that isotope labelling studies demonstrated their operation in sequence, this study uncovered no evidence of moonlighting but perhaps indicates a more important role of protein localisation in the regulation of primary metabolism than previously thought. Analysis at the subcellular level has largely followed analogous approaches to those described for the cellular level. Transgenesis approaches have been frequently adopted in which the gene of interest is fused to an organellar target sequence (see for example Farre et al., 2006; Wu et al., 2006; Bender-Machado et al., 2004; Sonnewald et al., 1991) in order to study the consequence of overexpressing an enzyme in a given organelle, while antisense and knockout approaches on the endogenous enzymes are also often carried out.

However, the reasons for the presence of metabolites in the absence of enzymes that catalyse their transformation remain more obscure. One example for which there is now strong cumulative evidence is the presence of sucrose in the plastid (Farre et al., 2001; Gerrits et al., 2001). Subcellular analysis of metabolite levels are difficult because metabolites cross organellar membranes incredibly rapidly and metabolism must therefore be quenched immediately prior to separation of the organelles. For this reason, subcellular analyses are generally carried out by taking one of two approaches. First described were techniques based on centrifugation. Two techniques are commonly used here: non-aqueous fractionation (Farre et al., 2001) and silicon oil centrifugation (Igamberdiev et al., 2001). We will concentrate here on the first, given the problems we highlighted above with working with protoplasts. That said, only silicon oil centrifugation is capable of resolving mitochondrial from cytosolic fractions. Non-aqueous fractionation methods were first applied to plant cells in the early 80s (Gerhardt et al., 1983), but have recently been readopted (Farre et al., 2001, 2006; Tiessen et al., 2002; Fettke et al., 2005; Lu et al., 2006). They work on the principle that metabolites stick to the membranes of the organelles which contain them and that these membranes exhibit different densities from one another. The application of GC–MS based metabolite profiling has greatly increased the information available from a centrifugal gradient and thus allowed estimates of a wider number of metabolite concentrations (Farre et al., 2001). Despite these advances, our knowledge of subcellular distributions of metabolites remains fragmentary at best. The second approach adopted to increase our understanding of metabolite compartmentation, is the use of genetically encoded metabolite sensors (Deuschle et al., 2006; Lalonde et al., 2005). This is highly advantageous in that it provides high temporal resolution of metabolite patterns, but can be difficult in plants due to the high levels of autofluorescence that they display and the limited dynamic range that these sensors afford. Another disadvantage of this method is that it is highly labour intensive and is only able to measure one metabolite at a time. Given the difficulties in directly assessing subcellular concentrations of metabolites, it appears likely that a combination of isotope labelling and modelling studies will be required to improve our understanding of the compartmentation of metabolism. Since the proteins present in the major organelles of the plant cell (not to mention the genomes and transcriptomes), have been relatively comprehensively catalogued (Heazlewood et al., 2007), a basis already exists for such models, which could be iteratively improved on each time innovations in data collection become apparent. The recent boom in bioinformatics-based research suggests that there should be enough interest to take on such a task. Whilst the complexity of the plant cell renders such modelling even more difficult, the current technical limitations under which we work suggest that following such a route will be imperative for the foreseeable future. In addition to the methods described above metabolic networks are beginning to be studied in plants by means of emerging techniques for protein–protein interaction studies (Noguera-Mazon et al., 2006).

Another research field that can be anticipated to gain yet greater prominence is that of the structural elucidation of proteins. Since the publication of the stuctures of the photosystems (Zouni et al., 2001; Jordan et al., 2001), the structures of a multitude of further proteins of secondary metabolism (Wang et al., 2006; Shao et al., 2005; Ma et al., 2006; Achnine et al., 2004; Zubieta et al., 2002), and a few of primary metabolism (Jin et al., 2005; Fieulaine et al., 2005; Unno et al., 2006) have been solved to a reasonable resolution. In addition, computer modelling of protein structure is becoming more and more apparent (see for example Pott et al., 2004). These studies allow for a much better understanding of metabolic regulation but also highlight key amino acid residues for enzyme functionality both in terms of binding constants and catalytic efficiencies and as such also have high potential for biotechnology needs to concentrate on in order to fully comprehend metabolic regulation.

7. Conclusion

The genomes of several photosynthetic species have been sequenced but vast gaps remain in our understanding of even the most basic of processes that they carry out. That said, the genome sequence provides a great tool that paves the way for much experimentation by approaches that could not have been imagined fifty years ago. The immediate future of phytochemistry is likely to be a case of more of the same and can be anticipated to concentrate of the identification of the chemical identity and biological role of secondary metabolites. This is only one research horizon that is apparent with work currently underway to understand better the role of metabolites in developmental (Palanivelu et al., 2003) and circadian (Dodd et al., 2005; Bläsing et al., 2005) processes as well as to understand more fully both the compartmentation and microcompartmentation of metabolism (Jorgensen et al., 2005) and biophysical aspects of metabolic regulation (see for

example Wang et al., 2006). However, just as we apply techniques inconceivable 50 years ago, we must accept – and indeed hope for – further developments in the next 50 years that are currently beyond our conceptual grasp.

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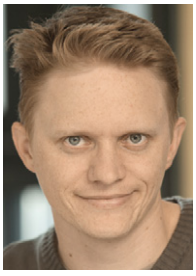

Alisdair Robert Fernie received his Bachelors degree in Biochemistry from Sheffield University before moving to the laboratory of Dr. Nicholas Kruger at the University of Oxford to study for his D.Phil on the completion of which he moved, in 1999, to the Max-Planck-Institut für Molekulare Pflanzenphysiologie in Potsdam-Golm. His work there was initially focussed on the metabolic engineering of starch accumulation in the laboratory of Prof. Lothar Willmitzer during which time he developed a keen interest in the emergent field of metabolomics. After his post-doctoral work, Alisdair co-led the Central Metabolism research group in Golm for a period of two years, following which he assumed full control of this group in 2003. Work in his group utilises a combination of genetic, molecular biological and biochemical approaches to better understand the metabolic regulation of primary and intermediary metabolism. Currently major research focuses are defining the function and importance of mitochondrial metabolism, resolving the genetic factors underlying crop compositional quality and establishing novel strategies aimed at elucidating pathway structures. In 2006, Alisdair was the recipient of the Phytochemical Society of Europe Pierre-Faber Award.