Metabolomics in plant environmental physiology

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Abstract

Changes in plant metabolism are at the heart of plant developmental processes, underpinning many of the ways in which plants respond to the environment. As such, the comprehensive study of plant metabolism, or metabolomics, is highly valuable in identifying phenotypic effects of abiotic and biotic stresses on plants. When study is in reference to analysing samples that are relevant to environmental or ecologically based hypotheses, it is termed ‘environmental metabolomics’. The emergence of environmental metabolomics as one of the latest of the omics technologies has been one of the most critically important recent developments in plant physiology. Its applications broach the entire landscape of plant ecology, from the understanding of plant plasticity and adaptation through to community composition and even genetic modification in crops. The multitude of novel studies published utilizing metabolomics methods employ a variety of techniques, from the initial stages of tissue sampling, through to sample preservation, transportation, and analysis. This review introduces the concept and applications of plant environmental metabolomics as an ecologically important investigative tool. It examines the main techniques used in situ within field sites, with particular reference to sampling and processing, and those more appropriate for use in laboratory-based settings with emphasis on secondary metabolite analysis.

Key words: Environmental metabolomics, flavonoids, HPLC, mass spectrometry, metabolites, phenolics, phenotype.

Introduction

The high variability of the natural environment presents great challenges for plants in terms of their capabilities to grow, compete with neighbouring plants, and respond appropriately to various abiotic and biotic pressures (Walters, 2004). Selection pressures relating to abiotic variation, such as changes in nutrient availability, irradiance, and temperature can vary enormously on spatial scales ranging from a few square metres to entire landscapes. In addition to this naturally occurring heterogeneity, anthropogenic influences on the environment such as atmospheric CO2 enrichment and nitrogen deposition create further environmental variation (Stevens et al., 2004). Phenotypic plasticity enables plants to withstand such environmental dynamism, within both short and long time scales, and is governed by genes which not only determine the character of an organism but also the degree of responsiveness of that character to environmental stimuli (Bradshaw, 2006). This phenotypic plasticity is measured by a plant’s ability to change the way they grow (morphological traits) and function (metabolic traits). There is wide variation in the degree to which these metabolic traits respond to environmental pressures, ranging from slight shifts in metabolite turnover such as that observed in responses to changes in C/N/P within the local environment (Rivas-Ubach et al., 2012), through to activation or upregulation of highly specialized...
metabolic pathways (Kusano et al., 2011). Over time, traits have been favoured through evolutionary processes of natural selection that are relevant to the range of conditions within species’ habitats and distributions. The understanding of the variation in these metabolic traits is critical to understanding of how the environment influences plant growth and how this manifests itself ecologically. In order to achieve this understanding of biochemical variation, plant physiologists have taken advantage of recent advances in analytical chemistry and bioinformatics, advances that has enabled the near-complete assessment of the biochemical composition of plant tissue. This approach is termed ‘metabolomics’ (e.g. Fiehn, 2002), and when in reference to analysing samples that are relevant to environmental or ecologically based hypotheses is termed ‘environmental metabolomics’ (Morrison et al., 2007, Bundy et al., 2009). Its applications broach the entire landscape of plant environmental physiology, from the understanding of plant plasticity and adaptation through to community composition and even genetic modification in crops. In recent years, a number of key reviews and future perspectives on this subject have provided an outline of how metabolomics can be utilized to aid the understanding and identification of natural environmental pressures and pollution effects on ecosystems (Miller 2007; Viant, 2008; Bundy et al., 2009; Penuelas and Sardans, 2009b; Macel et al., 2010; Sardans et al., 2011; Weckwerth 2011; Simpson et al., 2012).

The aim of this review is to introduce the main techniques for metabolomics research and the general applications of untargeted metabolite analysis to plant environmental physiologists who may be new to the field. It also focuses on the targeted analysis of individual metabolites with a special emphasis on carbon-based secondary compounds (CBSC), which have an essential role in plant-environment interactions.

Sample preparation and metabolite extraction: field- and laboratory-based approaches

The ability to identify and quantify a whole range of metabolites has over the past two decades has become progressively simpler and increasingly cost-effective. However, experimental design, controlled cessation of metabolism, and the subsequent extraction of metabolites from plant tissue remain the most crucial steps in carrying out a meaningful metabolomics study of the organism(s) in question (Saito and Matsuda 2010). To the beginner, Kim and Verpoorte (2010) describe in detail various metabolite harvesting and extraction techniques; Hines et al. (2008) provide a excellent assessment of metabolomes obtained in the field versus those from laboratory samples; and the introductory book on metabolome analysis by Villas-Bôas et al. (2007) gives a comprehensive overview of the analytical techniques suitable for metabolomics-based studies. In order to standardize the varied techniques and experimental designs for such studies, the Metabolomics Standards Initiative have provided guidelines for reporting metabolomic experiments (Morrison et al., 2007). The consensus in the above reviews is that it is essential to consider the purity of samples, to quench metabolism quickly, to use the most appropriate analytical equipment that is available, and to be able to sensibly interpret the large datasets. Each of those considerations is discussed below:

Organism purity and quenching metabolism

The availability of tissue should be considered at the start of the experiment. This will depend on the analysis pipeline, although typically 10–100mg fresh tissue is required for most analytical applications, namely gas chromatography (GC), mass spectrometry (MS), and nuclear magnetic resonance (NMR). The number of samples required for correct biological interpretation must also be considered; however, this number might be determined by the number of samples the researcher have access to, especially if rare or difficult-to-obtain plants are being analysed. The article by Broadhurst and Kell (2006) provides an excellent synopsis of the statistical considerations, including sample size, which is required for metabolomics experiments. It is also important to consider the purity of the samples being harvested for metabolite analyses: plant tissue which is contaminated with bacteria, fungi, or pests such as aphids may interfere with the results, especially if the samples are derived from natural habitats and not from controlled growth room facilities. The application of chemicals to control pests such as aphid and scarid flies in growth room facilities should also taken into account when studying the metabolome of plants exposed to such chemicals.

When sampling plant tissues for a metabolomic analysis, it is important to prevent the continuation of enzymatic and oxidative processes within cells as some metabolite turnover rates are less than 1 s. This ensures the metabolome remains unaltered and therefore represents an accurate ‘snapshot’ overview of the plant’s metabolism at the time of sampling. This is possible in the laboratory environment (ideally using liquid nitrogen or hot/cold organic solvents). Where sampling is required in the field environment, it is possible to use the methods of Ossipov et al. (2008) whereby leaf samples are frozen directly by total immersion in liquid nitrogen prior to transport to the laboratory for analysis. It is possible to freeze samples directly using liquid nitrogen contained within a ‘dry-shripper’, dry ice, or by placing the tissue in cold methanol. Where sampling is necessary within a marine environment, the methods of Goulitquer et al. (2012) circumvent many of the problems associated with extraction of metabolites from samples of high salt concentration. As the transportation of frozen samples from field to laboratory can be problematic in terms of cost and logistics, some metabolite analysis could be carried out on site. For example, the concentration of carotenoids, chlorophylls, and anthocyanins can be obtained via a basic solvent extraction of cut leaf discs followed by spectrophotometry on site (Sims and Gamon, 2002).

Extracting metabolites

Different solvents will extract different classes of metabolites: this is mainly dependent on the polarity of the metabolite
Although there are a variety of solvent systems and extraction procedures (Kim and Verpoorte, 2010), the most commonly used extraction procedure is based upon the methods of Overy et al. (2005) whereby methanol, chloroform, and water is added to frozen, homogenized tissue samples. The resultant bi-phasic solution is used for the analysis of polar metabolites such as amino acids and sugars contained within the uppermost water/methanol-based ‘aqueous phase’ and larger, non-polar metabolites such as lipids and chlorophylls are retained within the lower chloroform-based ‘organic phase’.

**Analysing metabolites**

There is a wide suite of technologies that can be employed to analyse the metabolites in the plant solvent extracts. The choice of analysis will depend on the availability of equipment but more importantly it will depend on the question or hypothesis being tested. The two main approaches are usually divided between metabolite fingerprinting and metabolite profiling. These approaches are distinct: metabolite fingerprinting is a semiquantitative technique with largely putative metabolite identification whereas metabolite profiling involves accurate chemical identification and quantification (Dunn and Ellis, 2005).

The metabolite fingerprinting approach has been a powerful technique for identifying general patterns in the metabolic response to stress or in population studies (Kunin et al., 2009; Field and Lake, 2011; Simpson et al., 2012). These fingerprinting techniques predominantly aim to analyse metabolites in a complex mixture by either directly injecting the crude extract into a mass spectrometer (a technique known as direct injection mass spectrometry; Dunn and Ellis, 2005; Walker, 2011) or by obtaining nuclear magnetic resonance (NMR) spectra of the whole mixture (Viant et al. 2003; Forseth and Schroeder 2011). The advantage of NMR fingerprinting over MS is there is less bias in the number and classes of metabolites that can be detected in NMR systems. This is because the detection is not dependent on the metabolites’ ability to ionize, and structural information can be obtained for unknown metabolites (Forseth and Schroeder 2011).

The resulting metabolite fingerprints should always be verified by quantitative metabolic profiling techniques (e.g. Davey et al., 2008). These profiling techniques separate the metabolites prior to identification either by GC, GC × GC techniques where multiple GC columns are combined to increase the separation, resolution, and data acquisition time per sample (Alnætter et al., 2012), or LC (see Arbona et al., 2009 for a review of the techniques). Of particular note is the protocol paper by Dunn et al. (2011) that describes the main assays for sample preparation, derivatization, and analysis by HPLC and GC-MS. Although Dunn et al. (2011) focus on human samples, these techniques can be directly applied to plant samples. Whichever separation approach is used, the metabolites within the extract will have to be identified either by their molecular mass, fragmentation pattern using MS, or chemical shifts using NMR. Viant and Sommer (2013), Forseth and Schroeder (2011), and Jaroszewski (2005) provide reviews on how these identification techniques can be used to assign function to the metabolites within an environmental studies context.

**Data interpretation and bioinformatics**

The large datasets that are derived from metabolomic studies are usually assessed, annotated, and visualized using curated online portals such as www.plantcyc.org (Zhang et al., 2010) and www.plantmetabolomics.org, where environmental and genetic information related to the experiments can also be considered as co-variables (Bais et al., 2010, 2011). Other project-specific sites also exist, such as the ECOMICS portal for metabolomics studies related to biomass production (https://database.riken.jp/ecomics/; Ogata et al., 2010) and the metabolomic network exchange (www.metnetdb.org). The large datasets can also be analysed by univariate and multivariate statistics such as principal component analysis using online servers such as Metaboanalyst (http://www.metaboanalyst.ca/; Xia and Wishart, 2011). These portals, and how metabolomic data can be incorporated into other omics, is reviewed by Bassel et al. (2012) and Mochida and Shinozaki (2011).

**Applications of metabolomics in plant environmental physiology**

**A matter of scale**

Metabolic approaches provide unique opportunities to examine the functional phenotype of individuals, and by doing so powerful insights into plant plastic responses to abiotic variation can be gained at the individual (Lake et al., 2009), population (Davey et al., 2008), and even community scales (Field and Lake, 2011). This can be achieved through the study of the metabolome and subsequent multivariate statistical analyses of species within a genus spread across many populations and locations. Recent examples of how metabolite fingerprinting can be used to study population dynamics include the study by Choi et al. (2010) in identifying the origin of coffee beans, Li et al. (2011) on their work on identifying populations of tobacco plants from China and Zimbabwe, and Davey et al. (2008) on identifying populations of the arctic-alpine plant Arabidopsis lyrata ssp. petraea from isolated regions across Europe. Follow-on research on the latter species by Kunin et al. (2009) showed that metabolomic variation was only detectable at the region scale (hundreds of kilometres apart) rather than at a within-region scale (ten of kilometres apart) and that there was no significant relationship between the metabolomic and genetic variation within a region. Such population studies are not limited to higher plants, as evidenced through the identification of shifts in the metabolomes of macro- micro-algal species associated with the time of day, sampling period, and geographic location by Riedl et al. (2012). Recent research by Scherling et al. (2010) also suggests that variation in the metabolome is a functional indicator for the competitive ability and subsequent biodiversity of plants within experimental plant communities. They found greater metabolic diversity in small herbaceous...
species compared to taller, more dominant plants within their experimental field plots. Also, the metabolic profiles indicated that carbon and nitrogen was limited in smaller plants when exposed to increasing diversity, indicating that changes in resource availability drive biodiversity. This is taken a step further by Field and Lake (2011) who revealed that metabolic diversity is directly linked with genotypic abundance within populations of wild plants. At an even larger scale, one of the most recent and exciting applications of metabolomics is the assessment of biochemical traits at a landscape scale (Asner and Martin, 2008; Asner et al., 2008). The development of airborne hyperspectral analysis, whereby changes in absorbance and reflectance patterns of specific chemical compounds within leaves is observed over entire landscapes using light aircraft and remote-sensing techniques, enables an estimation of biochemical phenotypes across relatively large scales.

Metabolomics can also contribute significantly to ecological questions and hypotheses on gross carbon, nitrogen, and phosphate allocation in plants, especially when the habitat is under environmental stress (Penuelas and Sardans, 2009a, b; Rivas-Ubach et al., 2012). For example, there are some excellent studies on the metabolic responses in plants to drought (Arbosa et al., 2010), ozone (Cho et al., 2008), ultra-violet light (Lake et al., 2009; Kusano et al., 2011), cold temperatures (Davey et al., 2009; Miyagi et al., 2010), and atmospheric nitrogen deposition in Lichens (Freitag et al., 2012). In a more applied context, metabolomics techniques have enabled measurement of metabolic responses to exposure to pesticides such as glyphosate (Petersen et al., 2011) and have been used in a variety of studies to look at drought tolerance in model plants and crops (Wilson et al., 2009; Bowne et al., 2011). As well as abiotic pressures, plants experience a number of biotic interactions within their natural habitat. Building on the pioneering research by chemical ecologists such as Thomas Eisner and Jerrold Meinwald (e.g. Eisner and Meinwald, 1995), a number of fascinating studies have emerged to examine the metabolic responses and interactions of herbivory using newly emerging technologies such as ultra-performance liquid chromatography MS and NMR (Jansen et al., 2009; Prince and Pohnert, 2010; Forsteth and Schroeder 2011). This chemical ecology approach in metabolomics is not restricted to higher plants, as it has also been used to assess the chemical defence responses in red algae (Nylund et al., 2011).

Genotype versus environmental effects on the metabolome

A key area for metabolomic research applications is in the understanding of how much of the variation in the metabolome is influenced by inherent genetic factors, including maternal effects on seed quality, and by external environmental factors. To date, the results strongly indicate that the environment affects the metabolome far more than genetic variation alone. This has been described by Robinson et al. (2007), who studied the metabolome of Douglas fir trees and found that metabolic (carbohydrate and lignin biosynthetic metabolites) and phenotypic (tree growth, fibre morphology, and wood chemistry) traits were strongly related to site, while similar associations relating to genetic (family) structure were less so. Also, the studies by Frank et al. (2009) on maize and Matsuda et al. (2012) on rice have shown that variation in the plant’s metabolome due to changes in the plant’s environment (such as growing location, seasons) was far greater than the variation caused by differences between strains or genetic modifications.

Targeted metabolite profiling: carbon-based secondary compounds

The remainder of this review is focused on the current techniques for identifying and quantifying low-molecular-weight CBSCs such as phenylpropanoids and isoprenoids. Secondary metabolites serve ‘essential’ functions in the interaction of a plant with its environment (Kutchan and Dixon, 2005). This large group of phytochemicals are utilized for many functions within the cell but the broad consensus is that many secondary metabolites are aimed at maintaining cellular redox homeostasis in response to changes in environmental conditions by inhibiting the generation of reactive oxygen species (ROS) and quenching ROS once they are formed, thus behaving as antioxidants (Vickers et al., 2009; Agati et al., 2012; Ramel et al., 2012). For example, isoprene biosynthesis is an early response to drought, where it quenches $O_2$ and confers thermal stability to chloroplast membranes, thus reducing oxidative damage (Vickers et al., 2009). Isoprene emission is severely constrained during severe drought and recent experiments have shown that zeaxanthin may indeed replace isoprene in maintaining rigidity of thylakoid membranes and possibly in quenching $O_2$ (Beckett et al., 2012; Ramel et al., 2012). Biosynthesis of zeaxanthin from $\beta$-carotene, which is a very effective chemical quencher of $O_2$ (Ramel et al., 2012) but increases membrane fluidity (Havaux, 1998), indeed confers drought tolerance (Davison et al., 2002; Du et al., 2010). Interestingly, recent evidence shows that flavonoids located in the chloroplast outer envelope membrane (OEM) may also quench $O_2$ and preserve the integrity of OEM under severe photo-oxidative stress, thus avoiding ROS-induced programmed cell death (Agati et al., 2007, 2013). Targeted metabolic profiling, using solvent extractions and spatial imaging of the metabolite in planta, will therefore help unravel the functional roles of individual secondary metabolites in plant–environment interactions. These extraction and analytical procedures are discussed below.

Extraction, identification, and quantification of CBSCs

The extraction of isoprenoid-based photosynthetic pigments (namely carotenoids) is performed with organic solvents miscible with water, such as acetone or tetrahydrofurane on plant tissues flash-frozen in liquid nitrogen after sampling and stored at –80 °C until analysis (Khachik et al., 1992). The addition of Ca- or Mg-carbonate (Lichtenthaler and Buschmann, 2001) or the organic base N-ethylisopropylamine (Kramer et al., 2003) is recommended to neutralize vacuolar acids. Pigments
such as carotenoids are usually separated and identified by reversed-phase HPLC (C$_{18}$ or C$_{30}$ columns) with a photodiode array detector (PDAD) (Mabry et al., 1970; García-Plazaola and Becerril, 1999; Lashbrooke et al., 2010). The HPLC-PDAD technique can also be used to separate, identify, and quantify phenolic metabolites such as simple phenylpropanoids and more complex phenolics such as flavonoids. Flavonoid extraction can be performed using acidified methanol or ethanol-based solvents to allow the extraction of both aglycones and flavonoid glycosides, as well as anthocyanins if present in the tissues (Merken and Beecher, 2000; Heier et al., 2002; Naczk and Shahidi, 2006). The phenolics can then be purified using solid-phase extraction or liquid/liquid extraction (e.g. with n-hexane) to remove lipophilic compounds. Insoluble wall-bound phenylpropanoids can be determined following the alkaline hydrolysis of the insoluble residue from the ethanol/methanol extractions (Strack et al., 1988). Even more difficult to extract are surface flavonoids, both those secreted by glandular trichomes and those present in non-secretory trichomes. These are analysed by mechanically removing trichomes or by quickly dipping the leaves in fresh chloroform or diethyl ether (Gould et al., 2000; Tattini et al., 2000, 2007).

Fig. 1. Multispectral fluorescence microspectroscopy and microimaging of a Ligustrum vulgare leaf exposed to full solar irradiance. A 100-µm-thick cross-section was stained with Naturstoff reagent (0.1 % 2-amino ethyl diphenyl boric acid in phosphate buffer, pH 6.8, with addition of 1 % NaCl) and fluorescence was recorded using the following λ$_{exc}$/λ$_{em}$ set ups: 365±5/480±5nm for hydroxycinnamates (HCA), 436±5/580±10nm for flavonoids, and 436±5/680±10nm for chlorophyll visualization (Agati et al., 2002, 2007). Image merges from the combination of 480/580/680nm fluorescence images. (A–D) Fluorescence spectra were recorded by a diode array multichannel spectral analyzer through an optical fibre bundle coupled to an inverted epifluorescence microscope. A total of 15 fluorescence spectra were recorded (integration time of 1 s) per tissue layer on a 0.0078 mm$^2$ spot moved along the horizontal axis of the cross section stained with Naturstoff Reagent and excited at 365nm (Agati et al., 2002). (E–H) Identification of individual flavonoids and HCA in different tissue layers by HPLC-PAD. Spectra in A and B and chromatograms in E and F derive from the analysis of adaxial epidermis and palisade parenchyma, in which flavonoids (particularly quercetin and luteolin derivatives) but not HCA accumulate (maximum emission at 575nm, consistent with the emission spectra of luteolin and quercetin derivatives; please also note the negligible contribution of the blue band at 480nm used for HCA visualization). Spectrum in (C) and chromatogram in (G) refer to the spongy parenchyma: flavonoids are in trace amounts whereas HCA largely accumulate (peak of maximum fluorescence at 540nm, consistent with the fluorescence spectra of glycosyl derivatives of caffeic acid at 530nm; note the substantial contribution of fluorescence at 480nm). Spectrum in (D) and chromatogram in (H) derive from abaxial epidermis in which flavonoids predominate over HCA (maximum fluorescence at 565nm). Que 3-O-rut, quercetin 3-O-rutinoside (rutin); Lut-7-O-glc, luteolin 7-O-glucoside; Api 7-O-gly, apigenin 7-O-glucoside and apigenin 7-O-rutinoside.
The metabolites mentioned above are non volatile, but for biogenic organic volatile compounds (BVOCs), such as volatile isoprenoids, sample collection is the crucial step for the successful metabolite analysis. Real-time analysis of BVOCs through proton-transfer-reaction mass spectrometry (PTR-MS, Lindinger et al., 1998) couples a fast detection of BVOCs (response time 1–100 s) with high sensitivity (detection limit of 10–100 pptv), and is particularly suitable for the detection of BVOCs mixtures whose concentrations change fast. Since the PTR-MS utilizes a soft chemical ionization of volatile organic compounds (Lindinger et al., 1998) it is difficult to distinguish between isobaric compounds (i.e., molecules of the same nominal mass but different accurate mass). To overcome this inherently low accuracy of PTR-MS, different chemical ionization reagents are required (CIRMS techniques, Blake et al., 2006) or the proton transfer reaction needs to be coupled with a TOF-MS (Jordan et al., 2006). BVOCs can be conveniently acquired from plants in the field using a portable IRGA system clamping a leaf in the gas-exchange cuvette, with the flow of contaminant-free air diverted into a tube packed with a suitable adsorbent phases. A variety of adsorbent phases are currently available depending on the individual BVOCs to be identified. The plant BVOCs trapped on adsorbing matrices are then thermally desorbed and assayed by GC-MS electron ionization (Tholl et al., 2006).

Inter- and intracellular distribution of CBSC using fluorescence microspectroscopy imaging

Phenolics, particularly flavonoids, have a prominent role in the mechanisms adopted by plants to respond to a wide array of environmental stimuli (Agati and Tattini, 2010). This is because the dynamic environment has forced plasticity within flavonoid metabolism, resulting in a range of metabolites located in different cells and subcellular compartments (Kutchan and Dixon, 2005; Agati et al., 2012).

Fluorescence-based techniques have long been used to detect different phenolics in a wide range of leaf tissues, including surface organs (Hutzler et al., 1998; Tattini et al., 2000, 2007; Agati et al., 2002). Fluorescence microspectroscopy enables estimating the tissue-specific distribution of different phenolic classes, at qualitative and semiquantitative levels (principles given in Agati et al., 2002). This is carried out by connecting a diode array multichannel spectral analyser to an inverted epifluorescence microscope and recording the fluorescence spectrum of UV-excited specimen excited with ‘fluorescent’ probes (usually aqueous NH3 or 2-amino ethyl diphenyl borate, Naturstoff reagent (Hutzler et al., 1998; Agati et al., 2002; Tattini et al., 2004, 2005)). The distribution of different polyphenol classes may be estimated, this technique alone is unable to detect the tissue-specific location of individual metabolites, mainly because the fluorescence quantum yields steeply differs and the fluorescence spectra of different metabolites strongly overlapping (Agati et al., 2002, 2009). Furthermore, phenolic glycosides (glycosylation allows phenolics to become soluble in the aqueous cellular milieu) that do not have a catechol group in the benzene ring are not fluorescent after reaction with the Naturstoff reagent (Shehan et al., 1998; Agati et al., 2009).

One solution to this problem is to obtain the identification and quantification of individual phenolics in different tissues by cryo-sectioning tissues (Ålenius et al., 1995) followed by HPLC analysis of the individual cell layers. An example of this technique is reported in Fig. 1 where fluorescence spectra (Fig. 1A–D) of different tissues of...
sun-acclimated *Ligustrum vulgare* leaves with the relative composition in individual phenolics (using HPLC analysis, Fig. 1E–H) are shown. The imaging shows that that in the epidermal (Fig. 1A, D, E, H) and palisade (Fig. 1B, F) tissues, derivatives of the flavonoids quercetin and luteolin dominate, whereas hydroxycinnamates are in very low concentrations. This suggests that flavonoids are unlikely to serve primarily as UV-B attenuators in photoprotection, as hydroxycinnamates have much greater molar extinction coefficients (ε) than quercetin and luteolin over the 280–315 nm spectral region (Harborne and Williams, 2000; Tattini et al., 2004; Agati et al., 2013). In contrast, flavonoids are in trace amounts in the inner spongy tissues, where hydroxycinnamates largely predominate (Fig. 2C, G). This further suggests that each cell tissue (possibly even a single cell) is autonomous in phenolic biosynthesis and that dihydroxy B-ring-substituted quercetin and luteolin derivatives serve as primary antioxidant functions in cells exposed to severe light-induced oxidative stress (Landry et al., 1995; Tattini et al., 2000; Agati et al., 2009, 2011; Agati and Tattini, 2010).

Recently developed multispectral fluorescence microimaging also allows the detection of different classes of metabolites using various different narrow-band excitation and emission wavelengths. This excitation/emission setup enables the selective and contemporary excitation of different metabolites and avoids the overlap of their emission responses (Tattini et al., 2004; Agati et al., 2007). Multispectral fluorescence microimaging when performed in confocal laser scanning microscopy allows the visualization of the subcellular location of phenolic metabolites (Fig. 2). Here dihydroxy ‘antioxidant’ flavonoids, in addition to being present in the vacuole of mesophyll cells, are located in the chloroplast and nucleus in leaves adapted to full solar radiation. The nuclear location of flavonoids, particularly flavonols is intriguing, and consistent with control exerted by flavonoids on the transcription of genes required for growth and development (Saslowsky et al., 2005; Pollastri and Tattini, 2011).

**Conclusions**

In light of this review of the applications and techniques for the small molecule profiling of plants, it is concluded that metabolomics has a valuable and functional future for environmental and ecological plant sciences. Not only will the equipment and techniques become more available to a wider range of scientists, the information acquired in these studies will enable a step-change in understanding how a wider range of plants interact with each other, with other species, and with the environment. It is also suggested that in plants experiencing vastly different environmental stimuli CBSC may serve multiple functions at different levels of scale, not only because the environment has conferred plasticity to specific metabolic pathways, but also because CBSC have very different inter- and intracellular locations.

**References**


