Metabolomics of forage plants: a review

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Introduction

Major forage plants of grazed pastures are the temperate grasses *Lolium perenne* (perennial ryegrass) and *L. arundinaceum* (tall fescue), and the legumes *Trifolium repens* (white clover) and *Medicago sativa* (lucerne, alfalfa). To increase the success of pasture-based animal production systems, forage breeding has focused on traits such as increased pasture production, tolerance and persistence under biotic and abiotic stress, and on improved forage quality (Humphreys and others, 2006; Parsons, 2011). Reduced opportunities to increase forage production solely through on-farm management such as fertilization or defoliation and feed allocation (Clark and others, 2007), as well as pressures to reduce negative environmental impacts (Abbott and others, 2008) require the development of new cultivars with superior agronomic traits as rapidly as possible.

Traditional plant breeding is based on phenotypic selection and subsequent progeny testing commonly followed by re-selection, which can be a very slow process and requires often time-consuming and costly phenotyping. To speed up the process and to make it more precise, quantitative trait loci (QTL) analysis to identify genetic markers which are used for marker-assisted selection (MAS) is being developed as a new tool for forage breeding (Zhang and others, 2006; Mouradov, 2010), but so far these genotypic markers have been mainly correlated to readily observable phenotypes, e.g. seed yield. Many desired traits are directly linked to specific metabolites, e.g. high sugar grasses to fructans (Turner and others, 2006), or insect resistance to certain secondary metabolites (Clay and Schardl, 2002); targeted analysis of these metabolite classes can be sufficient for selection and QTL analysis, although these methods are usually slow and not amenable for screening of large populations. However, the link to a desired phenotype can also be more complex, e.g. in the case of plant growth or drought tolerance where a complex profile of metabolites represents a chemotype related to the desired characteristics.
To unravel these profiles and to screen large numbers of plant genotypes and populations, metabolomics approaches have been developed to analyse a wide range of metabolite classes simultaneously and in a high-throughput and untargeted manner (Sumner et al., 2003; Hall, 2006; Fernie and Schauer, 2008; Langridge and Fleury, 2011). Other ‘-omics’ technologies such as genomics, transcriptomics or proteomics analyse only one type of chemical compounds, i.e. DNA, RNA or proteins, and require generally only a small number of analytical platforms. Metabolomics, on the other hand, is faced with a multitude of chemical structures with physicochemical properties ranging from highly hydrophilic (e.g. sugars) to hydrophobic (e.g. fatty acids), as well as a large dynamic range from molar to nanomolar (e.g. some alkaloids) to even lower (e.g. some phytohormones) concentrations. Currently no technology is available which gives a truly ‘holistic’ view of the metabolome, and a combination of several extraction, separation and detection techniques is required to give an overview of most metabolites present in a sample. The main instrumentation used is based on mass spectrometry (MS) coupled to gas chromatography (GC) and/or liquid chromatography (LC), but other technologies such as nuclear magnetic resonance (NMR) or capillary electrophoresis coupled to MS are also used. All of these technologies produce very data-rich outputs and require extensive processing (e.g. noise removal, peak alignment, peak picking and deconvolution, peak identification) and mining (e.g. multivariate statistics, machine learning, network analysis) techniques to deliver meaningful information which needs to be interpreted and utilized in the plant breeding process. It is beyond the scope of this review to discuss these techniques in detail and we refer to Hall (2011) and references therein for further reading.

Through providing a broad analysis of a plant’s chemical composition, metabolomics approaches can provide a significant opportunity for plant breeders to assess for traits which have previously been considered too difficult or too expensive to include in breeding programmes. Such traits have required the development of specific targeted analyses or the application of a significant amount of effort by skilled labour, which would provide a barrier to their routine assessment. In addition, these approaches are implemented more broadly and integrated with other high-throughput genomic technologies, they will enable plant breeders to assess several of these characteristics concurrently. However, setting up the appropriate instrumentation and bioinformatics infrastructure still requires large financial inputs often beyond the means of small institutes or breeding companies especially in the area of forages. The majority of metabolomics studies so far have therefore focused on the analysis of model plant species by academic institutions to develop the technologies, or cereal crop and vegetable species with larger financial margins for the breeding industry. For examples of successful implementation of metabolomics, together with other molecular breeding tools in potato, tomato, rice and other fruits and cereals, we refer here to Stewart et al. (2011) and references therein. In comparison, the use of metabolomics for forage species and its implementation in forage breeding programmes still lags behind and we will review here those few studies which have analysed the metabolome (or parts of it) in forages.

**METABOLOMICS ANALYSIS OF FORAGE PLANT RESPONSES TO ABIOTIC STRESS**

Major challenges for pasture production arise from the reduced availability of three resources, i.e. water, nitrogen (N) and phosphorus (P). Plants have developed an array of adaptation mechanisms to cope with low or fluctuating availability of these resources, most of which result in reduced vegetative growth. The majority of detailed studies of molecular and metabolic responses to resource limitation has been performed with either model (mainly *Arabidopsis thaliana*) or cereal crop species with a major focus for the latter on seed yield. Intensive animal production on pastures, however, requires high vegetative plant yield, and much more research on how forage plants respond to low resource supply and how these responses might be manipulated is needed.

**Metabolic responses to osmotic stress**

Extended periods of drought pose a serious challenge to agricultural production systems including pastures. Global climate change has been predicted to increase the variance of climatic parameters, which may lead to unpredictable and more intense events of drought (Gornall et al., 2010), and many breeding programmes are directed to develop forage and crop cultivars which survive severe drought better and/or continue to be high yielding under moderate drought conditions (Wilkins and Humphreys, 2003; Humphreys et al., 2006). Improved survival and/or growth characteristics under drought can be achieved by plants through a multitude of strategies (Orcutt and Nilsen, 2000; Chaves et al., 2003) each involving different sets of traits (Ludlow and Muchow, 1990; Richards, 1996, 2006; Collins et al., 2008). Furthermore trade-offs between increased drought adaptation and productivity are to be expected, and these will depend on how well cultivars with changed strategies cope with a variety of climate scenarios (Tardieu, 2005; Sambatti and Caylor, 2007). To be successful in overcoming at least some of these complex problems requires a much better understanding of plant responses to drought (and other abiotic stresses) on the molecular level, but only a very few studies have been performed on forages compared with crops (Zhang et al., 2006). For example, metabolic and transcript profiling of *Lolium perenne* exposed to PEG-induced water stress compared responses of a susceptible genotype from the cultivar ‘Cashel’ to a genotype from an accession classified as ‘drought tolerant’ (Foitio et al., 2009). The main outcome of that study was that a large number of metabolites responded to water stress with amino acids, fatty acids and phytosterols generally decreasing, while sugars and organic acids increased. Major differences between the two genotypes were related to much higher increases in quinic and shikimic acid, phytosterols, raffinose and trehalose in the drought-tolerant accession. This general trend of increases in sugars and organic acids and decreases in amino acids was also found in a comparative metabolomics study of model and cultivated *Lotus* species subjected to either severe short-term or to moderate long-term drought (Sanchez et al., 2011b).

Quinic and shikimic acids are precursors for phenylpropanoids such as flavonols, and studies on *Trifolium repens*...
subjected to abiotic stresses such as UV irradiation (Hofmann et al., 2001), drought (Hofmann et al., 2003) and cold (Rasmussen et al., 2006; Rasmussen, 2009) showed a strong increase of flavonols, particularly quercetin conjugates. Cultivars and ecotypes of T. repens with constitutive high levels of flavonols and anthocyanins had also been shown to be more UV and drought tolerant (Hofmann et al., 2001, 2003), indicating that high levels of phenylpropanoid pathway metabolites might protect plants from abiotic stress (Dixon and Paiva, 1995). However, it has also been shown that there is no simple correlation between levels of stress-related metabolites and stress tolerance (Ashraf and Foolad, 2007; Sanchez et al., 2011), and selection for high-flavonoid clovers has not resulted in more stress-tolerant cultivars so far. Higher levels of some compatible solutes (e.g. proline, glycine betaine), usually interpreted as an indication of increased stress tolerance are in fact observed in some drought-sensitive genotypes of barley and potato (Chen et al., 2007; Vazquez-Robinet et al., 2008; Widodo et al., 2009). Only one-quarter to one-third of metabolite responses were shared between the Lotus model and cultivated species, demonstrating that findings from studies with model species cannot be simply translated to cultivated species (Sanchez et al., 2011b).

Metabolic responses to osmotic stresses are often related to common changes in carbon availability, usage and growth (Hummel et al., 2010), but a comparison of metabolic responses to drought and salt stress in Lotus species showed that only one-third to one-half of metabolic responses were conserved, while some metabolites, particularly organic acids, responded in the opposite direction, i.e. were increased under drought but decreased under salt stress (Sanchez et al., 2011, 2012). Clearly, more research, particularly on forage cultivars, is needed to identify robust metabolic traits and pathways conferring drought tolerance to improve chances of success of current breeding efforts, and this research should be integrated with other -omics data, whole plant physiology and genetics (Cattivelli et al., 2008; Salekdeh et al., 2009; Roy et al., 2011).

**Metabolic responses to nutrients**

Plants require large amounts of nitrogen for optimal photosynthesis and vegetative growth, and green tissues of forage grasses in highly N-fertilized pastures contain up to 4.5% total N (Parsons et al., 1991). N is taken up as ammonium or nitrate from soils, with nitrate being the major form (Crawford and Glass, 1998). Nitrate acts as a signal inducing a range of metabolic enzymes involved in carbon and amino acid metabolism, while it reduces flavonoid biosynthesis (Lam et al., 1996; Sakakibara et al., 2006; Krouk et al., 2010). A comprehensive study comparing the metabolic composition of L. perenne plants grown at high-N versus moderately low-N supply, resulting in 4% versus 3% total N content in blades, showed significant differences in a large number of metabolites (Rasmussen et al., 2007, 2008a). As expected, strong effects were seen for nitrogenous compounds of which the majority were much higher at high-N supply. However, major amino acids (e.g. glutamine and aspartate), which represented 85% of the total free amino acids, were much more affected than minor amino acids, which was in accordance with other studies (Noctor et al., 2002). Nitrate assimilation is strongly linked with photosynthesis and carbon metabolism (Stitt et al., 2002; Smith and Stitt, 2007) and was reflected by a shift from carbohydrate to organic acid and lipid biosynthesis (Rasmussen et al., 2008a). This study also tested the effects and interactions of resource supply and infection of Lolium perenne with an endophytic Neotyphodium lolii fungus, which produces alkaloids protecting its host from insect herbivory, and one of the major findings was that high-N supply resulted in reduced accumulation of alkaloids produced by this fungus, which will be discussed in the section ‘Ryegrass–endophyte associations’.

Phosphorus is the second most important mineral nutrient essential for plant growth and low-P availability severely limits crop and pasture growth (Vance et al., 2003). P fertilization is becoming increasingly expensive as natural resources of phosphate rock are dwindling (Gilbert, 2009; Vance, 2001). Phosphate deprivation results in a complex re-programming of metabolism, and plants have evolved four major adaptation strategies involving changes in root morphology, improved P uptake, increased P recycling and improved P economy (Vance et al., 2003; Morcuende et al., 2007; Müller et al., 2007; Nilsson et al., 2010). A transcriptomic and metabolic profiling study on early response mechanisms of L. perenne to P starvation revealed an enhanced uptake of sulfur and mobilization of glycerol 3-phosphate, which is indicative of a remodelling of membranes from phosphor lipids (to release P) to sulfolipids (Byrne et al., 2011) as well as seen in A. thaliana as well (Missen et al., 2005). Other effects were a genotype-specific reduction of sugars, amino acids, fatty acids and organic acids in leaves while sugars and some fatty acids were increased, indicating effects on photosynthesis (Hammond and White, 2008; Byrne et al., 2011). Improved efficiency of P use can be achieved by activating glycolytic bypasses (Theodorou and Plaxton, 1993) and Byrne et al. (2011) presented evidence that this strategy can be employed by L. perenne. A high level of transcripts coding for cellulose synthases accompanied with reduced expression of xylanases was interpreted by the authors as indicative of remodelling of cell walls; however, it is unclear how P deficiency and cell wall biosynthesis are interconnected (Byrne et al., 2011).

Plants have evolved additional strategies to cope with a low nutrient supply, i.e. legumes can form symbiotic associations with dinitrogen-fixing rhizobial bacteria, while both grasses and legumes can also form associations with arbuscular mycorrhizal fungi (AMF) to improve P solubilization and uptake. Metabolomics studies of these associations will be reviewed in the following sections.

**METABOLOMICS ANALYSIS OF SYMBIOTIC FORAGE PLANT ASSOCIATIONS**

Like many other plants, forages form a wide variety of associations with endosymbiotic heterotrophic microbes. One of the most important is the association of legumes such as T. repens (white clover) and M. sativa (alfalfa, lucerne) with rhizobia as these provide pastures with additional nitrogen through fixation of atmospheric dinitrogen (N₂) gas (Graham and Vance 2000, 2003). Common to both forage grasses and...
legumes are associations with AMF which facilitate the solubilization and utilization of immobilized soil phosphorus (Read and Perez-Moreno, 2003; Cappellazzo et al., 2008; Smith and Read, 2008). A symbiotic association specific to cool-temperate grasses such as L. perenne and L. arundinaceum is the infection with fungal *Neotyphodium/Epipichloë* spp. endophytes which provide their host plants with anti-herbivorous alkaloids (Leuchtmann, 1992; Clay and Scharl, 2002; Christensen et al., 2008). In all cases the heterotrophic microorganisms depend on carbon and energy provided by their autotrophic host plants. How a mutualistic co-operation (both partners benefit) between the symbiotic partners is maintained over the course of evolution and especially how the partners prevent each other from ‘cheating’ (only one partner benefits) is still debated, but control of metabolite/nutrient exchange between the partners seems to play an important role (Kiers and Denison, 2008; Ryan et al., 2008; Draper et al., 2011).

Metabolomics approaches have been used to study the effects of these associations on metabolic composition of the whole symbiotum or specialized cell structures like arbuscules and nodules, and will be discussed in the following section.

**Associations of forage legumes with rhizobia**

Mineral soil nitrogen is a major limiting factor for pasture-based animal production requiring the application of nitrogen fertilizers to intensive production systems. Global market prizes for these fertilizers have steadily increased over the past decades due to high energy consumption in the production process, and environmental impacts due to nitrate leaching and nitrous oxide emissions from pastures have renewed an interest in biological nitrogen fixation by legumes. Major forage legumes in pastoral systems are *Trifolium* and *Lotus* species, and alfalfa (lucerne) which are either grown in mixed swards with forage grasses for grazing or as monocultures for hay and silage production. Rhizobial bacteria infect legume roots and form nodular structures which are able to fix otherwise inaccessible atmospheric N₂. The establishment of successful nodulation and active N-fixation depends on a complex signalling pathway which involves metabolite (e.g. isoflavonoids, phytohormones, lectins) -based communication between the two partners (Matamoros et al., 2006; Gibson et al., 2008; Oldroyd and Downie, 2008; Ding and Oldroyd, 2009).

Primary metabolism undergoes major changes during the transition from nodule-free root tissues to functional nodules and it has been proposed that malate is the major source for carbon and energy for nodule-inhabiting bacteroids (Vance et al., 1994; Schulze, 2004). An induction of genes related to sucrose degradation, glycolysis, phosphoenolpyruvate carboxylation and malate synthesis increasing malate production in nodules, and will be discussed in the following section.

**Associations of forage plants with AMF**

Phosphorus is the second major limiting factor for productivity in pastoral systems and, due to dwindling reserves and increasing costs of natural P fertilizers, solutions are needed to increase available soil P. Arbuscular mycorrhizal (from the Greek words for ‘fungus’ and ‘root’) fungi (AMF) play a critical role in the acquisition of inorganic phosphorus from insoluble P sources in soils and transfer to their hosts (Read and Perez-Moreno, 2003; Cappellazzo et al., 2008; Smith and Read, 2008). Like rhizobial bacteria, AMF are also heterotrophic and completely dependent on carbon and energy supply by the host. Up to 20 % of fixed carbon can be transferred to AMF, contributing considerably to the carbon sequestration capacity of soils (Bago et al., 2000; Zhu and Miller, 2003).

Successful infection and arbuscule formation requires a complex re-programming of root cells which is strikingly similar to that seen in nodule formation (Akiyama and Hayashi, 2006; Besserer et al., 2006; Oldroyd et al., 2009; Soto et al., 2010; Draper et al., 2011; Gaude et al., 2012). Metabolomics studies of AMF associations are very rare and most of the current knowledge of metabolite exchange and metabolism is based on detailed studies employing labelled isotopes and NMR spectroscopy (Bago et al., 2000, 2001; Pfeffer et al., 2001). In the developed symbiosis, carbon is translocated as hexoses to the arbuscules and intraradical fungal hyphae and subsequently metabolized to trehalose, glycogen and lipids. Lipids are translocated to the extraradical hyphae where they are used for fungal growth and maintenance. Extraradical hyphae can take up P from the soil in the form of inorganic phosphate and translocate it as
Some of the alkaloids produced by grass endophytes such as lolitremes and ergovaline are toxic to grazing livestock (Gallagher et al., 1984; Lyons et al., 1986; Strickland et al., 1996), and a range of fungal genes and gene clusters responsible for alkaloid biosynthesis have been identified (Spiering et al., 2002; Tanaka et al., 2005; Young et al., 2006; Fleetwood et al., 2007). Major efforts are under way to identify novel endophytic strains with beneficial metabolic profiles, and high-throughput analytic methods employing direct infusion MS proved to be useful for rapid screening of large numbers of infected seeds and plants (Koulman et al., 2007) and identified a previously unknown class of cyclic oligopeptides produced by endophytes (Cao et al., 2008). Moreover, a study testing the effects of nitrogen supply, high-sugar ryegrass cultivars and strains of endophytes differing in their alkaloid profile revealed that several additional metabolites apart from alkaloids and particular combinations of these were relevant for insect responses to infected plants (Rasmussen et al., 2008b). The above studies also demonstrated that endophyte alkaloid production and endophyte concentrations depended strongly on the ryegrass cultivar (Rasmussen et al., 2007, 2008a; Liu et al., 2011), supporting the findings that the host-plant genotype regulates fungal growth (Eaton et al., 2002), and a genetical metabolomics study described in the following section identified several QTL in the ryegrass genome relevant to these traits (Koulman et al., 2009).

GENETICAL METABOLOMICS AND FORAGE BREEDING

The combination of genetics and metabolomics, also known as genetical metabolomics has been successfully used in model and crop species to identify genetic regions related to specific metabolites or metabolic profiles (for reviews, see Keurentjes et al., 2006; Keurentjes, 2009; Kliebenstein, 2009; Fernie and Klee, 2011). An untargeted metabolomics and QTL analysis of 2000 mass peaks detected in a recombinant inbred line of Arabidopsis thaliana allowed the reconstruction of the glucosinolate pathway, confirmed that at least two QTL contribute to variation in aliphatic glucosinolates, and uncovered new biosynthetic steps related to flavonol glycosides (Keurentjes et al., 2006). Other studies of this type identified QTL for flavour volatile emissions in tomato (Schauer et al., 2006; Tieman et al., 2006) and flavonols in poplar (Morreel et al., 2006).

Only a very few studies have been published on genetical metabolomics in forage plants and we will discuss two examples here. A targeted metabolic profiling approach has been used to identify QTL related to high sugar content in blades of L. perenne (Turner et al., 2006). Grasses with higher sugar levels in blades have been bred to increase energy supply to the rumen microorganisms, decreasing N losses from rumen protein degradation and increasing N supplied to the ruminant, thereby reducing N losses in urine and nitrous oxide emissions from pastures (Miller et al., 2001; Wilkins and Lovatt, 2003; Edwards et al., 2007; Parsons et al., 2011a, b). Temperate grasses accumulate water-soluble fructans, i.e. polymeric chains of fructose attached to a core molecule of sucrose, which are an alternative reserve carbohydrate

polyphosphate to the intraradical hyphae and arbuscules, and eventually to plant cells (Schachar-Hill et al., 1995; Javot et al., 2007). Metabolic profiling of Glomus intraradices-infected M. truncatula (a model legume closely related to M. sativa) roots revealed levels of some amino acids, fatty acids, isoflavonoids and tyrosol in arbuscular relative to uninfected root tissue (Schliemann et al., 2008). This study also identified the fungal-specific metabolites trehalose, Δ1-unsaturated fatty acids, sterols and apocarotenoids. Interestingly, trehalose was also identified as a rhizobium-specific metabolite in the above-mentioned study on B. japonicum-infected root hairs in soybean (Brechenmacher et al., 2010).

Ryegrass–endophyte associations

Cool temperate forage grasses such as L. perenne and L. arundinaceum can also be infected by clavicipitaceous fungal endophytes which reside exclusively in the apoplastic spaces of above-ground plant parts and do not form any kind of specialized symbiotic compartments (Leuchtmann, 1992; Clay and Schardl, 2002; Christensen et al., 2008). These endophytes produce a variety of alkaloids which have antiherbivorous activities and protect the host plants from grazing mammals, insects and other invertebrates (Bush et al., 1997; Lane et al., 2000; Schardl et al., 2004). Unlike for rhizobia and mycorrhiza, not much is known about signaling processes between endophytes and their hosts, but a role for reactive oxygen species and possibly iron for the regulation of fungal growth in planta has been demonstrated (Tanaka et al., 2006, 2008; Koulman et al., 2012). Transcriptomic analyses of host plant responses to endophyte infection have been hampered by the lack of available genome sequences; however, the genome sequences of several Epichloë strains have recently been made publicly available (http://www.endophyte.uky.edu/). A study using high-throughput mRNA sequencing showed that disruption of a fungal kinase leads to dramatic changes in gene expression profiles associated with proliferative fungal growth, early plant senescence and down-regulation of fungal secondary metabolites, indicating a shift of the nature of the association from mutualistic to pathogenic (Eaton et al., 2010).

Neotyphodium/Epichloë spp. endophytes are completely dependent on micro- and macronutrient supply by their host plants, but very little is known about the mechanisms of exchange of metabolites, and no specific sugar or amino acid transporters have been functionally characterized so far (Draper et al., 2011). Targeted and untargeted metabolomics analyses comparing infected with uninfected plants show a significant decrease in nitrogenous compounds (e.g. nitrate, asparagine, proline, proteins) and some fibre components with a concomitant increase in soluble carbohydrates and organic acids such as malate, quinate, shikimate and phenylpropanoids (Cao et al., 2008; Rasmussen et al., 2008a, 2009a). The effects on sugars, specific organic acids and stress-related metabolites seems to be a common feature of all three symbiotic associations discussed here, and are in fact common to most biotrophic, including pathogenic interactions (Draper et al., 2011).
to starch (Pavis et al., 2001). Biosynthesis and accumulation of fructans show marked seasonal variation (Pollock and Jones, 1979), and mobilization of fructans plays an important role for re-growth after defoliation (Morvand-Bertrand et al., 2001). Fructan metabolism is very complex and considerable interactions of the expression of the high sugar trait in high-sugar grass cultivars with temperature, duration of re-growth after defoliation, nutrient supply, flowering and association with symbiotic microorganisms has been shown (Pollock and Cairns, 1991; Parsons et al., 2004; Rasmussen et al., 2007, 2009b; Liu et al., 2011). Turner et al. (2006) mapped fructan, sucrose, glucose and fructose contents in L. perenne leaves and tiller bases using an F2 mapping family from a high water-soluble carbohydrate (WSC) × low WSC cross in two seasons (spring and autumn) over 4 years. Despite high variability of the traits and considerable environmental variation, significant genetic effects were detected and the broad-sense heritability was moderate to high. The metabolic QTL (mQTL) explained between 8% and 59% of the total phenotypic variation with the largest effects for fructan and total WSC QTL near the top of chromosome 6. However, a large proportion of the total phenotypic variation was unexplained by QTL, and many QTL were identified in only 1 year, probably reflecting the strong impact of environmental conditions on the expression of the trait (Kamoshita et al., 2002). A subsequent study analysing relationships between growth and drought-stress QTL with fructan QTL (Turner et al., 2008) showed that correlation between growth and fructan content was low to negative suggesting that growth is likely to be related to a not very well-defined inherent plant growth capacity rather than to carbohydrate storage. Recently developed LC–MS-based methods for the rapid analysis of polymeric fructans up to DP 100 (degree of polymerization) revealed that high sugar grasses mainly differed in large polymeric fructans (Harrison et al., 2009, 2011, 2012). These high-DP fructans are difficult to separate and quantify with the analytical methods commonly used for fructan analysis and MS analysis might reveal additional and larger QTL for high sugar grasses.

An untargeted direct infusion MS/MS analysis of metabolites in clonal replicates of 200 genotypes of an endophyte-infected L. perenne F1 mapping population from a pair cross between two heterozygous genotypes in two consecutive years identified 22 ions with one or more reproducible QTL across 2 years (Kouman et al., 2009). QTL were detected for the fungal alkaloid peramine and for an unknown fungal cyclic oligopeptide identified in a previous study (Cao et al., 2008), clearly showing that fungal metabolite production is at least partially regulated by the host genome and suggesting that marker-assisted selection might be an option for breeding of endophyte–host associations with a particular metabolic make-up. A large number of ions with QTL were unknowns and would need more targeted LC–MS/MS analytical methods and purification for compound identification. Two of the unknowns were subsequently isolated and shown to be the plant pyrrolizidine alkaloids thesinine-rhamnoside and a thesinine-rhamnoside-hexoside which had not been described previously in Poaceae (Koulman et al., 2008). Some pyrrolizidine alkaloids have been associated with insect-deterring activities (Frölich et al., 2006), and the finding that thesinine accumulated in most of the ryegrass cultivars tested lead the authors to speculate that breeders have inadvertently bred for this metabolic trait (Koulman et al., 2008). This clearly shows that untargeted metabolomics is very powerful in detecting unknown metabolic consequences of breeding.

Another promising area for implementing metabolomics into forage breeding is the development of MS-based analysis of grass-fibre composition. Low digestibility (or degradation rate) of grass cell walls can negatively affect feed intake and fibre chemical composition has been targeted in some forage breeding programmes (Casler et al., 2008). The measurement of in sacco or in vivo degradation rates of plant material is expensive and not useful for screening individual plants in a breeding programme. Analysis of plant cell-wall composition and the correlation of concentrations of extracted wall compounds such as etherified hydroxycinnamic acids with degradation rates has therefore been proposed as an alternative approach (Lam et al., 2003). However, cell walls in grasses are a complex of insoluble polyphenolic lignin tightly linked to hemicellulose and cellulose by esterified and etherified hydroxycinnamic acids. Current extraction and detection methods for these fibres are difficult to use, time-consuming and costly, which has hampered the development of new cultivars with improved digestibility characteristics. Recently, a more mild extraction method with subsequent chromatographic separation of larger complexes of lignin, polysaccharides and hydroxycinnamic acids and detection by MS has been described (Faville et al., 2010). This method is amenable to high-throughput analysis of large sample sets, and relatively high correlation (up to 0.63) with in sacco degradation rates was seen for some of the unidentified cell-wall polymers. These compounds also showed considerable genotypic variation and might be very useful as metabolic markers in future forage breeding programmes. A study of seasonal changes in metabolic composition of 21 grass and legume cultivars using NMR also revealed high correlation of sugar concentrations, fibres and in vitro organic matter digestibility with the NMR fingerprints, mainly due to differences in spectral intensities of malic acid, choline and glucose (Bertram et al., 2010). These studies highlight that metabolomics-based approaches deliver high-throughput detection and selection methods for forage genotypes with improved feed quality and can help overcome some of the current limitations of forage breeding programmes.

**CONCLUSIONS**

Meeting current and future demands of the pastoral industry for new forage cultivars with superior agronomic characteristics to increase animal production and reduce environmental impacts will be a challenge for plant breeders. New technologies such as genome sequencing, transcriptomics and metabolomics can be very powerful tools for large-scale geno- and phenotyping, and for improving our understanding of complex traits and our ability to manipulate them. Particularly the combination of genetics and metabolomics technologies is a very promising, albeit under-developed technique for forage breeding. The plant metabolome is very complex and the development of metabolomics approaches is still in its early stages and requires currently expensive analytical instrumentation and extensive bioinformatics
 infrastructure. This has resulted in slow uptake of these technologies by forage breeders and a lack of examples of successful implementation in forage breeding programmes so far.

Much of the knowledge of the traits discussed in this review comes from studies on model species or cultivated cereals. These plants are most often inbreeding annually with flowering and seed setting as a major strategy for survival of the population. Forage species, on the other hand, are mostly outbreeding perennials which have evolved long-term strategies for survival and propagation of vegetative material often involving extended phenotypes, i.e. associations with symbiotic microorganisms. Furthermore, the harvested component of forage plants in grazed pastures is vegetative photosynthetically active tissue, the only source of new carbon for plant growth and so pasture production. Hence, yield is a trade-off between removing leaves to feed grazing animals while sustaining enough leaf material to photosynthesize, and improvements in ‘yield’ are very difficult to achieve and sustain for grazed forage plants. These special features have limited the success of translational approaches from model species and more research on major forage species is urgently needed to assist forage breeding in overcoming current limitations of pasture-based production systems. To be most effective, these techniques need to be accompanied by whole-plant physiology, proof of concept (modelling) studies related to the efficacy of specific traits, and wider considerations of possible consequences of new traits, particularly on fitness of new populations.

**LITERATURE CITED**


