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Screening for potential co-products in a *Miscanthus sinensis* mapping family by liquid chromatography with mass spectrometry detection

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ABSTRACT

Society is demanding more green chemicals from sustainable sources. *Miscanthus* is a potential source of platform chemicals and bioethanol through fermentation. *Miscanthus sinensis* (*M. sinensis*) has been found to contain particularly high levels of soluble phenols (hydroxycinnamates and flavonoids) which may have application in the nutraceutical, cosmetic and pharmaceutical industries. Here, we describe the first study on the identification and quantification of phenols from the leaf tissue of a bi-parental *M. sinensis* mapping family. Parents and progeny showed complex profiles of phenols with highly related structures which complicated characterisation of individual phenotypes. Separation of semi-purified extracts by reverse-phase liquid chromatography, coupled with detection by diode array and ESI-MS/MS, enabled distinction of different profiles of phenols. Ten hydroxycinnamates (*O*-cinnamoylquinic acids) and several flavones (one mono-*O*-glycosyl flavone, eight mono-*C*-glycosyl flavones, two di-*C*-glycosyl flavones, five *O*-glycosyl-*C*-glycosyl flavones and nine 2″-*O*-glycosyl-*C*-glycosyl flavones) were identified and quantified in leaf tissue of two hundred progeny and maternal and paternal plants during the seedling stage. Progeny exhibiting high, moderate and low amounts of hydroxycinnamates and flavonoids and both parents were selected and screened at seven months' growth to determine the abundance of these phenols at their highest biomass and compared with seedlings. Concentrations of phenols generally decreased as leaves matured. Several flavone-glycosides were identified. This technique can be used for rapid screening of plants in a mapping family to identify genotypes with high phenol content to add value in the biorefinery chain. This comparative study provides information on the content of potentially valuable compounds from readily renewable resources and possible biomarkers for identification in breeding programmes.

1. Introduction

Society is becoming increasingly concerned about the negative impact on the environment associated with the chemical industries and this has led to a demand for the supply of "green" chemicals from sustainable sources. The identification of high-value chemicals within energy crops would increase value in the biorefinery chain, which could increase the adoption of bioenergy and reduce dependence on fossil fuels. A biorefinery using plant-based feedstocks could generate multiple products, including platform chemicals, fuel and power (Cherubini, 2010). Advances in cleaner processing technologies, particularly in fermentation, molecular and genetic engineering, have increasingly allowed chemical industries to use plant-based, as opposed to petrochemical, feedstocks for the manufacture of commercially important chemicals (Xu et al., 2008). Green chemicals are not limited to the fuel sector but are also relevant to a range of commercial sectors, including the pharmaceutical, nutraceutical, cosmetic, food and beverage industries (Dong et al., 2011; Crozier et al., 2009; An et al., 2008; Aburjai and Natsheh, 2003). The world production of biomass each year is thought to exceed 1×10^{14} kg (Xu et al., 2008). In the US alone, 2.5×10^{11} kg of the plant biomass produced each year is wasted and exceeds the current total consumption of 1×10^{11} kg used for the production of organic chemicals, plastic resins and fibres (Xu et al., 2008). Moreover, only 5% of chemicals are presently sourced from renewable resources and there may be potential for bio-chemicals to share markets with their petrochemical-based counterparts (Xu et al., 2008).

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There is huge interest in using perennial grasses, such as *Miscanthus* spp. and *Panicum virgatum*, as a source of renewable biomass (Lewandowski et al., 2000). Components of biomass, such as lignin, cellulose and hemicellulose (Kleinert and Barth, 2008), are used to provide bioenergy in the form of heat, electricity and liquid fuels (Cherubini, 2010). There are numerous other chemicals with potential commercial value present in grasses (Heaton et al., 2008). *Miscanthus* is a good source of biomass for biorefining, owing to its high lignocellulose yield, producing nearly three times more harvestable biomass at cool temperatures (3×10^4 kg hectare^{-1}) than does *P. virgatum* (1×10^4 kg hectare^{-1}) (Heaton et al., 2008). In addition, *Miscanthus* is able to tolerate marginal lands and flooding (Heaton et al., 2008; Lewandowski et al., 2000). Biomass from *Miscanthus* has a high lignin content, comprising around 24–25% of the cell wall, making it closer to a woody material than to a grass (Lygin et al., 2011; Villaverde et al., 2010). Furthermore, hydroxycinnamic acids (specifically ferulic acid and *para*-coumaric acid) are covalently linked into the cell wall of grass species, where they serve to cross-link hemicellulose and lignin by ester and ether bonds, and their concentration is thought to be correlated with ease of hydrolysis of the biomass (Akin, 2007).

Plants produce a wide array of mono- and poly-phenols, with roles in strengthening cell walls, UV protection, stress tolerance and resistance to pathogens (Parveen et al., 2010, 2011). These include hydroxycinnamic acid conjugates and flavonoids from the phenyl propanoid and shikimate pathways and have antioxidant and UV-absorbing properties. The identification of commercially important molecules from crops to maximise value within a biorefinery has resulted in a demand for high-throughput screening tools for the extraction, identification and quantification of bio-chemicals. High performance liquid chromatography (HPLC) is the most widely used analytical technique for the characterisation of a wide range of polyphenolic compounds. LC-electrospray ionisation-tandem mass spectrometry (LC–ESI-MS/MS) has been used to characterise and quantify rapidly hydroxycinnamic acid conjugates and flavonoids in a wide range of plants (Parveen et al., 2011; Valls et al., 2009; Clifford et al., 2006).

Miscanthus is currently being investigated as a biofuel plant; however, little work has been carried out to identify high-value chemicals in this perennial grass. Mapping families are produced by crossing parents with contrasting phenotypes for a selected trait. The progeny are then screened for variation (segregation) in the trait which can then be associated with differences between the progeny in genomic sequence. The overall aim of this study was to screen a mapping family to identify commercially important chemicals which increase the value in the biorefinery chain and to identify genotypes and growth stages which show highest abundance of these compounds. Examples are luteolin and its glycosides, which are reported to have anti-melanogenic activity (An et al., 2008), radical-scavenging activity, anti-inflammatory activity (Odontuya et al., 2005) and antibacterial activity (Dillard and German, 2000).

Characterisation of individual progeny is complicated by complex profiles of phenols and a high abundance of related structures and the objective was to determine the application of LC-PDA–ESI-MS/MS for detecting variation within the mapping family. The soluble phenol profiles in the leaf tissue of a biparental *Miscanthus sinensis* mapping family (two hundred progeny and both maternal and paternal plants) were investigated in the early development growth period (at one month's growth) and at their highest biomass at seven months' growth. We discuss the variation that exists for high-value chemicals that may be used to develop a genetic screen for such chemicals.

2. Results and discussion

A *Miscanthus sinensis* (*M. sinensis*) mapping family, consisting of a bi-parental cross, was selected for study due to its high phenotypic variability. The maternal plant exhibited a stay-green trait, while the paternal plant produced high biomass and high numbers of seeds. LC-DAD–ESI-MS/MS, in negative ion mode, was used to screen two hundred progeny and maternal and paternal plants for soluble phenols in early developmental growth (one month seedlings). More than thirty hydroxycinnamates and flavonoids were identified and quantified in leaf tissue of progeny and both parents (Fig. 1). This method proved effective for distinguishing differences in profiles of phenols between progeny. The progeny were divided into categories (low, medium and high) according to their content of hydroxycinnamates and flavonoids (Fig. 2). Eleven progeny exhibiting high, moderate and low concentrations of hydroxycinnamates and flavonoids and both parents were selected and screened at seven months' growth to determine the abundance of these soluble phenols at their peak of production of biomass and their levels were compared with their profiles of phenols when seedlings. Information on how the content of phenols within the leaf tissue differs between the early and late periods of growth may provide insight into how value may be increased within a biorefinery chain. Ratios of the hydroxycinnamates to flavonoids on a weight-per-weight basis were higher in seedlings (73:60) and lower in mature leaves (12:18). Phenols are known to be synthesised in the cytoplasm and chloroplasts and, as the plant matures, phenols accumulate in cell vacuoles or polymerise into lignin to strengthen the secondary cell walls (Kefeli et al., 2003).

The results showed that the progeny varied significantly in their concentrations of hydroxycinnamic acid conjugates, flavonoids and total phenols in seedlings and mature leaves (Fig. 3). In general, the levels of the phenols decreased as the leaves matured. This is not surprising, as seedlings contain only a primary cell wall and, as the secondary cell wall thickens in mature tissue, soluble phenols are incorporated into lignin (Kefeli et al., 2003). This is also in agreement with Martin, who investigated the effect of stage of maturity of perennial ryegrass on phenolic compounds and found that increasing maturity of the herbage was linearly correlated to the decrease in the proportion of soluble phenols in leaf tissue and increase in the lignin content of the herbage (Martin, 1970).

A significant decrease was observed in the concentration of total phenols from one month to seven months' growth, with the most abundant sample decreasing from 8.0 mg/g FW to 0.1 mg/g FW. Plants synthesise phenols as a protective mechanism against herbivores, microbial pathogens, invertebrate pests and hostile environmental stresses (Parveen et al., 2010). To date, studies on the effect of maturation of the leaf on the concentrations of polyphenols are scarce. Although there was a decrease in the total content of phenols of the paternal plant from 5.3 mg/g FW to 2.1 mg/g FW and a reduction in the maternal plant from 1.6 mg/g FW to 1.1 mg/g FW, this may suggest that differences in the total contents of phenols of parents are less marked after seven months' growth, in comparison to the seedling stage, and may indicate that soluble plant phenols may be rapidly lost in the first few weeks of development. Interestingly, ca. twenty-five percent of the progeny had a profile of phenols similar to that of the paternal parent, while seventy-five percent showed a profile similar to that of the maternal parent. This population represents the F1 progeny of a biparental cross and the 3:1 ratio may indicate that the chemical composition is inherited in a classical Mendelian fashion and that the paternal phenotype is linked with recessive alleles for genes involved in the expression of this trait. If the chemical phenotype were also associated with simple Mendelian segregation of markers, it would allow for the robust association of genetic markers

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