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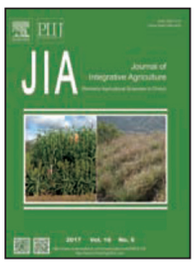

RESEARCH ARTICLE

The application of Fourier transform mid-infrared (FTIR) spectroscopy to identify variation in cell wall composition of *Setaria italica* ecotypes

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Abstract

Cell wall composition in monocotyledonous grasses has been identified as a key area of research for developing better feedstocks for forage and biofuel production. *Setaria viridis* and its close domesticated relative *Setaria italica* have been chosen as suitable monocotyledonous models for plants possessing the C_4 pathway of photosynthesis including sorghum, maize, sugarcane, switchgrass and *Miscanthus×giganteus*. Accurate partial least squares regression (PLSR) models to predict *S. italica* stem composition have been generated, based upon Fourier transform mid-infrared (FTIR) spectra and calibrated with wet chemistry determinations of ground *S. italica* stem material measured using a modified version of the US National Renewable Energy Laboratory (NREL) acid hydrolysis protocol. The models facilitated a high-throughput screening analysis for glucan, xylan, Klason lignin and acid soluble lignin (ASL) in a collection of 183 natural *S. italica* variants and clustered them into classes, some possessing unique chemotypes. The predictive models provide a highly efficient screening tool for large scale breeding programs aimed at identifying lines or mutants possessing unique cell wall chemotypes. Genes encoding key catalytic enzymes of the lignin biosynthesis pathway exhibit a high level of conservation with matching expression profiles, measured by RT-qPCR, among accessions of *S. italica*, which closely mirror profiles observed in the different developmental regions of an elongating internode of *S. viridis* by RNASeq.

Keywords: monocotyledonous C_4 grasses, lignin biosynthesis, Fourier transform mid-infrared spectroscopy, *Setaria italica*

1. Introduction

Setaria has been proposed as a suitable C_4 monocotyledonous model for detailed investigation of Type II cell wall biosynthesis, composition and deconstruction (Petti *et al.* 2013; Martin *et al.* 2016). Within the Panicoideae clade, *Setaria* is closely related phylogenetically to a number of key potential biofuel feedstocks, including sorghum, maize, sugarcane, switchgrass and *Miscanthus×giganteus* (Brutnell *et al.* 2010). Although significant advances have been made using other representatives of monocotyledonous grasses, including members of the genus *Oryza* and *Brachypodium* (Rancour *et al.* 2012; Guo *et al.* 2014), these models possess the C_3 mechanism of photosynthesis. There is a correlation for the mechanism of photosynthesis, either C_3 or C_4, having an influence on monocot cell wall composition

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and plant species possessing these alternate mechanisms respond differently to environmental stresses such as drought, which may in turn impact upon photoassimilate partitioning towards soluble or structural polysaccharides (Doust *et al.* 2009; Petti *et al.* 2013). Furthermore, C_4 forage grasses exhibit more extensive lignification and recalcitrance to ruminant digestion (Norman *et al.* 2009). The genomes of two closely related species of *Setaria* have been sequenced in entirety, *Setaria italica* (foxtail millet) and *Setaria viridis* (green foxtail) with *S. italica*, the domesticated species grown commercially, whereas *S. viridis* is reportedly the wild progenitor (Doust *et al.* 2009). *S. viridis* however is considered a more tractable target for genetic manipulation primarily due to its smaller stature.

In grasses, the primary cell wall is comprised of 20 to 35% cellulose, whereas the secondary cell wall possesses a higher proportion of up to 50% on a dry weight basis. The major classes of hemicelluloses present in monocot cell walls are xylans (principally glucoronoarabinoxylan; GAX), mixed linkage glucans (MLGs) and xyloglucan (XIG), with GAX making up between 20 to 40% of the primary cell wall and between 40 and 50% of the secondary cell wall. The hydroxycinnamates, ferulic acid and *p*-coumaric acid comprise 1–5% of the primary cell wall and up to 1.5% of the secondary cell wall. The lignin polymer makes up 20% of the secondary cell wall (Vogel 2008). A negative correlation between lignin content and hydrolysis of plant cell wall material in either a ruminant or second generation biofuels context, has been widely reported (Norman *et al.* 2009; Fu *et al.* 2011). Lignin is considered to restrict access of water and saccharification enzymes to the cell wall increasing the cost and reducing yield potential of bioenergy crops (Slavov *et al.* 2013).

Improving digestibility for increased saccharification of bioenergy crops is currently being implemented through breeding, mutation or genetic manipulation strategies (Xin *et al.* 2008; Mullet *et al.* 2014; Wang *et al.* 2016). Mutations induced in C_4 crop species such as sorghum or maize either spontaneously or through chemical application or radiation exposure, have produced a swathe of lines exhibiting reduced lignin biosynthesis (Xin *et al.* 2008, 2009). The brown mid-rib phenotype (bmr), clearly evident in the leaves of sorghum plants with reduced lignin, reflects a mutation in discrete enzymes catalysing steps in lignin biosynthesis. For example, *bmr6* and *bmr12* have mutations in the cinnamyl alcohol dehydrogenase (*CAD*) and caffeic acid O-methyltransferase (*COMT*) genes respectively (Bout and Vermerris 2003; Sattler *et al.* 2009; Saballos *et al.* 2012).

Identification of lines exhibiting the desired cell wall phenotype in large populations, whether it be reduced or altered lignin composition or some other significant cell wall component, is most efficiently implemented through the application of high-throughput screening strategies. Such strategies have been implemented as efficient enzymatic assays (Chundawat *et al.* 2008; Santoro *et al.* 2010) or more rapidly and economically through the use of vibrational spectroscopy techniques including near infrared spectroscopy (NIRS) (Haffner *et al.* 2013; Wolfrum *et al.* 2013), Raman spectroscopy (Lupoi *et al.* 2014, 2015), and in this case, Fourier transform mid-infrared (FTIR) spectroscopy.

FTIR measures the absorption of infrared light by vibrational modes within molecules, making it directly diagnostic of the molecular composition of a sample. FTIR analysis of plant cell walls has been used for at least 40 years (Morikawa and Senda 1978) and was first used as a screening tool for *Arabidopsis* cell wall mutants 18 years ago (Chen *et al.* 1998). Since then, the availability of multivariate modelling techniques, including partial least squares regression (PLSR) and artificial neural networks (ANN), have allowed FTIR spectra to be calibrated with wet chemistry data in order to rapidly quantify the chemical composition of a sample (Duarte *et al.* 2002; Irudayaraj and Tewari 2003). Furthermore, FTIR has also been incorporated into a holistic screening strategy for biofuel feedstocks, capable of quantifying cell wall digestibility and soluble sugar content of *Sorghum bicolor* for a rapid, economical assessment of lines in a field setting (Martin *et al.* 2013). Herein, this approach (Martin *et al.* 2013) has been extended to generate PLSR models for the prediction of cell wall components in a diverse collection of *S. italica* ecotypes with diverse global origins.

2. Materials and methods

2.1. Plant materials

A collection of 183 *S. italica* accessions was obtained from the Australian Grains Genebank (AGG) (http://www.seedpartnership.org.au/associates/agg) and grown in a naturally illuminated glasshouse at the University of Newcastle, Australia (Latitude –32.93, Longitude 151.76) from February to July 2013. Temperature conditions were controlled at (24±1.5)°C during the day and (16±1.5)°C during the night. Accessions were planted in a soil mix containing equal amounts of perlite, coir peat and coarse river sand. A week after germination Osmocote high K® was applied, followed by weekly treatment with 1:1 mix of Osmocote Standard®. During the vegetative growth phase, phenotypic differences were monitored and upon harvest key measurements such as biomass, plant height, stem length and diameter as well as lifecycle length were recorded. At maturity, the leaves and sheaths were removed from the primary stem which was then subjected to compositional and FTIR spectral analysis. Following the first round screen of the *S. italica* collection, four accessions were selected for further analysis based on differences in their physical