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Lignin biosynthesis in sugarcane is affected by low temperature

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ABSTRACT

Sugarcane is an abundant and promising source of plant biomass for biofuel production. The use of biomass for conversion to ethanol is limited by the recalcitrance of lignocellulosic material mainly due to the presence of lignin. This study characterised some aspects of lignification in the stem of two sugarcane genotypes grown under low (CT) and warmer (HT – reference) temperatures. Stems were separated in young and mature culms and the culms were separated in cortex (rind) and medulla (pith). Plants of the genotype IACSP04-627, which has more lignin in the stem, grew better (fresh weight of the stem) than IACSP04-065 under HT. Cold negatively affected plant growth but apparently IACSP04-065 was more sensitive than the other genotype. Lignin content was significantly increased in the young rind of IACSP04-627 plants at CT, what could not be directly correlated with the expression profile of genes of the monolignols biosynthesis. Lignin content in mature rind was reduced in IACSP04-065 plants exposed to CT, what could be correlated with the low expression level of the genes *ShCAD2*, *ShCOMT1* and *ShCCoAOMT1*. The high expression of *ShF5H* occurred preferentially in mature pith of both sugarcane genotypes, what is possibly related with the early formation of the secondary cell wall induced by low temperature. In conclusion, lignin deposition in sugarcane under low temperature seems to be differentially regulated in rind and pith tissues and it is genotype-dependent.

1. Introduction

Sugarcane is the main source of bioethanol in Brazil, which is produced from the fermentation of sucrose-rich juice obtained after stem crushing. The production of sugarcane bioethanol is significantly more efficient than ethanol production using other sources, such as corn (*Zea mays* L.), beet (*Beta vulgaris* L.), and corn stover, which need to de-branch the starch before fermentation (Goldemberg et al., 2008). This ethanol is known as first-generation ethanol.

Despite the high sucrose content in sugarcane juice, two-thirds of the total sugars stored by the plant remain in the industrial residues of sugarcane (bagasse and straw) and are located mainly in the cell walls of stems and leaves as structural carbohydrates (Soccol et al., 2010). The energy stored in cell wall carbohydrates (cellulose and hemicellulose) has high potential for the production of cellulosic ethanol (also known as second-generation or lignocellulosic ethanol), however, such use of biomass requires a pre-treatment to disrupt the cell wall in order to release sugars in fermentable form (Himmel et al., 2007; Yuan et al., 2008). The main constituents of lignocellulosic biomass are cellulose, hemicellulose and lignin, with the relative proportions of the three components varying according to the material's origin (Reddy and Yang, 2005). Sugarcane bagasse consists of approximately 50% cellulose, 25% hemicellulose and 25% lignin (Pandey et al., 2000).

Abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; HCT, p-hydroxycinnamoyl-CoA:quinate shikimate p-hydroxycinnamoyl transferase; C3H, p-coumarate 3-hydroxylase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; F5H, ferulate 5-hydroxylase; COMT, caffeic acid *O*-methyltransferase; CAD, cinnamyl alcohol dehydrogenase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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Lignin has essential functions on plant growth and development because it enables water transportation at the xylem, increases cell wall resistance and rigidity, and acts as a natural barrier against insect and pathogen attacks (Iiyama et al., 1994). However, due to the chemical bonds established with cell wall polysaccharide network, lignin is one of the main factors associated with plant biomass recalcitrance, limiting the use of structural sugars for the production of bioethanol and other biomaterials (Chen and Dixon, 2007). Lignin restricts the efficiency of cellulose saccharification by acid pre-treatment, and especially by enzymatic hydrolysis (Chen and Dixon, 2007; DeMartini et al., 2013; Siqueira et al., 2011).

Sugarcane lignification progresses with the maturation of internodes, and the storage parenchyma begins to lignify at the same time that lignin deposition intensifies the thickening of the bundle sheath cells. This process is concomitant with the increase in sucrose storage at the parenchymatous tissue of mature internodes (Jacobsen et al., 1992). Subsequently, the epidermis and hypodermis also become lignified.

In sugarcane, lignin deposition at the cell walls is a spatially and temporally regulated process, varying between different plant parts, tissues and cell types (Bottcher et al., 2013). Lignin is a heteropolymer composed mainly of the *p*-hydroxycinnamyl alcohols (monolignols) *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, which form the *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, respectively, when they are incorporated into lignin (Boerjan et al., 2003). In sugarcane, the lignin content and composition (S/G ratio) are differentially regulated during stem development and at the two anatomically different regions of the internode, the cortex and the medulla (Bottcher et al., 2013).

Sugarcane is typically grown in tropical and subtropical areas. Therefore, temperatures below 20 °C may compromise its normal growth and cause damages to plant tissues (Moore, 1987). These negative effects may be explained by the absence of efficient cold resistance strategies, which are mainly related to the genetic basis of modern sugarcane cultivars, deriving greatly from *Saccharum officinarum*, a species that contributed to maximising sucrose accumulation but is sensitive to low temperatures (Khan et al., 2013).

Some studies have reported the effects of low temperatures on the biosynthesis of cell wall polysaccharides, e.g., in wheat (*Triticum aestivum* L.) (Zabotin et al., 1998) and *Arabidopsis* (Qu et al., 2011). Ford et al. (1979) studied the effects of temperature on cell wall metabolism in grass species from tropical and temperate climates. The authors observed that in tropical grasses, the cellulose content decreased and the hemicellulose and lignin contents increased with increasing temperature, whereas in temperate grasses, cellulose, hemicellulose and lignin contents increased with increasing temperature. Tolerance of *Miscanthus* sp. clones to low temperatures during cold acclimation is related to significant increases in phenylalanine ammonia-lyase and cinnamyl alcohol dehydrogenase enzyme activities (Domon et al., 2013). In corn mesocotyls, the increase in activity of two cell wall peroxidases and the higher lignin content during plant cold acclimation was related to tolerance mechanisms, which may be involved in the preservation of cell integrity in response to the damage caused by low temperatures (Anderson et al., 1995).

Considering the importance of sugarcane bagasse as a biomass source for the production of lignocellulosic ethanol, this study investigated the influence of low temperatures on lignin deposition in stems of two sugarcane genotypes with different lignin contents. Our results indicate that the tested sugarcane genotypes exhibit different levels of sensitivity to temperature variation and that lignin deposition in stems was affected differently. The possible implications for plant physiology and the quality of bagasse, and therefore bioethanol production, are discussed.

2. Materials and methods

2.1. Plant material

Two sugarcane (*Saccharum* spp.) genotypes, IACSP04-627 and IACSP04-065, originating from a progeny of 66 individuals undergoing selection for use as forage plants (M.G.A. Landell, unpublished data) were tested. Lignin contents for genotypes IACSP04-627 and IACSP04-065 were previously determined at 8.12% and 4.32%, respectively (Santos, 2014).

Twenty plants (10 per genotype) obtained from lateral buds (February 2010) of mother plants were grown in a greenhouse in 20 L pots containing commercial substrate and were watered daily. Secondary sprouts (tillers) were periodically removed, keeping only the main stem of each plant. Plants were divided into two groups at 110 days after planting. One group was grown at ambient (low) temperature (CT) inside the greenhouse and the other at higher temperature (HT) in a chamber located inside the greenhouse. The chamber consisted of a wood structure covered with clear plastic used to cover greenhouses. An oil heater was placed inside the chamber and was kept on between 5:00 pm and 9:00 am, increasing the temperature inside the chamber to approximately 8.3 °C above ambient temperature (Fig. S1). Plants grown at ambient temperature were therefore exposed to the low night temperatures of that time of the year in Campinas, state of São Paulo (SP), Brazil. Plants were grown under these conditions for 75 days, following which their tissues were collected for analyses. Therefore, collection was performed 185 days after the sprouts were transferred into pots. The experimental treatments were applied from 20th May 2010 to 20th August 2010 (Fig. S1). The average daily temperatures were 26.5 °C inside the chamber and 14.5 °C outside the chamber. At the end of the experiment, the stem was numbered from the apex towards the base, and the first internode (I1) was identified as the internode at which the leaf +1 was inserted. Internodes were separated between young (pool of internodes I1–I3) and mature (I11) internodes. Each sample was separated into cortex (rind) and medulla (pith). The material collected was immediately placed in liquid nitrogen and was subsequently divided into two parts. One part was freeze-dried and stored at −80 °C for biochemical analyses, and the other was stored at −80 °C and reserved for molecular analyses.

2.2. Biometric data

Prior to material collection for biochemical and molecular analyses, the following plant growth parameters were determined: number of green leaves, number of stem internodes, stem length, internode diameter (taken in the middle of the internode), stem fresh weight, leaf dry weight and root dry weight. Only leaves with more than 50% of the leaf blade exhibiting green colour were considered for determination of the number of leaves. Stem internode diameter was measured using a digital calliper. Roots were carefully washed under running tap-water with a sieve to avoid any possible root loss. Dry weights were determined following drying of the material in an oven at 72 °C.

2.3. Determination of soluble sugar contents

Freeze-dried pith samples were homogenised in a mortar, and approximately 30 mg of sample was extracted twice with 1 mL of 80% ethanol at 40 °C for 30 min. Following centrifugation, 750 μL supernatant was collected from each extraction, pooled together, and dried in a SpeedVac (Savant). Residues were dissolved in 1 mL milli-Q water, and 50 μL was diluted in 950 μL of 80% acetonitrile. Samples (4 μL) were analysed through mass spectrometry (MS) using an Acquity Ultra Performance Liquid Chromatography