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Short communication

Cuticular wax variants in a population of switchgrass (*Panicum virgatum* L.)

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ABSTRACT

Leaf cuticular waxes are known to influence both biotic and abiotic stress tolerances of plants. The objective of this work was to characterize the wax phenotypic diversity present in a population of 1849 switchgrass plants. We identified 92 visually distinct variant plants that possessed altered leaf glaucousness relative to the common standard type (ST), which exhibited a bluish-white (glaucous) leaf color. The variants could be grouped into three classes: 1) non-glaucous types (NG) that possessed a shiny green leaf surface, 2) reduced glaucous types (RG) that appeared less glaucous than ST, and 3) highly glaucous types (HG) that exhibited more intense bluish-white color than ST. Analyses of total cuticular wax content averaged over each of three NG (mean 304.79 ± 15.16 μg/dm^2), RG (mean 533.33 ± 21.62 μg/dm^2) and HG types (mean 1228.23 ± 45.74 μg/dm^2) showed significant differences ($P < 0.001$) from three selected STs (mean 810.92 ± 30.57 μg/dm^2). Analysis of wax composition among these selected types revealed that the C_{33} β-diketones were the most abundant wax compounds in all but NG types. Field emission scanning electron microscopy showed that abaxial leaf surfaces exhibited predominantly rod-shaped crystals, and adaxial surfaces exhibited predominantly plate-shaped wax crystals on all lines, except for NG that lacked wax crystals on the abaxial leaf surface. As a target for crop improvement, this study reveals that a large amount of variation for cuticle waxes exists within this switchgrass germplasm.

1. Introduction

The plant cuticle is a hydrophobic barrier that coats most aerial plant surfaces, and is composed of aliphatic compounds deposited both within and above the structural cutin matrix of the cuticle membrane (Kosma and Jenks, 2007). The cuticle plays a role in limiting plant transpiration and improving plant water conservation, which is especially important during climatological drought (Riederer and Schreiber, 2001). It also helps to protect the plant from insect herbivory, fungal pathogens and reduces potential heat stress by increasing reflection of solar radiation (Reicosky and Hanover, 1978; Jenks et al., 1995). The cuticular waxes on most plants are dominated by very long chain fatty acids (VLCFAs; ~C_{20}–C_{36}) and their derivatives including aldehydes, primary alcohols, alkanes, and the longer-chain length wax esters that can reach roughly 70 carbons long (Buschhaus et al., 2007). Other common wax classes include secondary alcohols, ketones and, in some species, β-diketones (von Wettstein-Knowles, 1972; Kunst and Samuels, 2003). When visualized by scanning electron microscopy, the epicuticular waxes are deposited as smooth coatings, or crystallize into a diverse array of amorphous, rod-like, or dendritic structures, depending on species and organ surface (Jeffree, 2007).

Tulloch and Hoffman (1980) identified the primary leaf surface wax components of switchgrass (*Panicum virgatum* L.) as C_{33} β-diketone (tritiacontane-12,14-dione) and a C_{33} hydroxy-β-diketone (5-hydroxytritriacontane-12,14-dione), accounting for 69% and 6% of the total wax load, respectively. β-Diketones have been identified in the wax of several members of the Poaceae including wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) (Hen-Avivi et al., 2016), and other non-Poaceae such as *Rhododendron* (Evans et al., 1975), and *Hosta* (Jenks et al., 2002). The current understanding of the impact that β-diketones have on glaucousness is previously described (Jeffree, 2007; Zhang et al., 2015; Hen-Avivi et al., 2016).

The glaucous trait, created by a visible deposition of epicuticular wax crystals, has been a target for research in several crop species (Zhang et al., 2015). Casler (2012) described the waxy bloom as a blue-colored (glaucous) coating on the stems and leaves of lowland ecotypes of switchgrass. In wheat, a similar bluish-gray leaf coating was linked to the presence of β-diketones and increased grain yields. Work to

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elucidate genetic control over the synthesis of β-diketones in wheat and barley revealed the involvement of the *Cer-cqu* gene cluster, which contains three independent genes in the β-diketone synthase polyketide pathway (Schneider et al., 2016). In barley, β-diketones are dramatically reduced in *cer-c* and *cer-q* mutants, but in *cer-u* a compensatory increase in β-diketones was observed in the absence of hydroxy-β-diketones (von Wettstein-Knowles, 1972). Switchgrass is a model herbaceous species for bioenergy production, and significant emphasis is now being placed on elucidating agronomically important traits and the underlying genes that determine biomass yield, including stress tolerance (Wright and Turhollow, 2010; Lowry et al., 2015). Characterization of existing variability in leaf wax structure and composition in switchgrass germplasm is an important first step toward understanding the underlying potential for this trait in future crop improvement strategies. In this report, a survey of the ultrastructure and chemical composition of leaf surface waxes existing on standard and glaucousness variants of the lowland ecotype (Alamo) switchgrass is presented.

2. Material and methods

2.1. Plant material and growth conditions

Seeds of lowland *P. virgatum* cv. Alamo were kindly provided by Dr. David Bransby, formerly of Auburn University. Seeds were treated with 80 mM EMS (ethyl methanesulfonate, Sigma-Aldrich, LLC, St. Louis MO) for four hours and rinsed several times. The initial interest in creating an EMS population was to screen, via bagging, for dominant mutations leading to self-fertility and apomixis. The EMS concentration and time for treatment were chosen from a dose response curve that was conducted. As no dominant mutants were observed, and there was no possibility of identifying recessive mutants in an obligate outcrossing population, the EMS treatment is not considered further in the report. After treatment, approximately 1000 seeds were planted in each of eight flats that were stratified for three days at 4 °C then transferred to a greenhouse. After three weeks, seedlings were transplanted into Ray Leach "cone-tainers" (SC-10 Super-White Low Density, Hummert International, Springfield MO). Before transplanting to the field, plants were trimmed to a height of 30 cm and hardened off by exposure to ambient temperature and humidity inside a screened headhouse. In March of 2011, plants were transferred to the field in an equally spaced (61 cm between plants and 91 cm between rows) grid containing 29 rows with between 47 to 81 plants per row. Field experiments were conducted at the University of Arizona's West Campus Agricultural Center (CAC) (32° 13′48″ N, 110° 57′11″ W; elevation 776 m). The West CAC field has a Vinton Soil Series (0–3% slope, fine sandy loam, Typic Torrifluvents) with irrigation water that is slightly alkaline and high in calcium (Weaver et al., 2014). This semi-arid climate (low relative humidity RH < 20%) has a bimodal annual precipitation that is concentrated during the summer monsoon months, July through September, and during the winter storm months of December through January. In 2011, Tucson observed an average yearly temperature of 69.9 °F and annual precipitation of 31.06 cm, which was similar to the previous 30-year averages of 69.4 °F and 29.34 cm, respectively (NOAA, 2014). In September 2011, variants were scored for plant height, stature, number of tillers, leaf width, leaf curling and plant color/reflectance. Among the 1849 transplants, most of the plants displayed a moderate amount of leaf glaucousness and are referred to as standard type (ST). 92 plants exhibited distinct visible differences in surface glaucousness when compared to the ST. The 92 variants were grouped into three distinct categories including three non-glaucous (NG), 14 reduced glaucous (RG), and 75 highly glaucous (HG) types. In addition, six plants were identified with extreme glaucousness on both leaf surfaces along with a very short stature and few tillers. These six plants were not considered further. From these primary visual screens, we selected three representatives from the ST, NG, RG, and HG types for a more detailed analysis of the glaucousness trait. For five successive growing seasons these individuals expressed their respective glaucous phenotypes. None of the glaucous variants, or any of the other phenotypes that were identified, was chimeric as would be expected if the mutagenesis treatment contributed to any of the observed phenotypes in the M1 population. As such, the variation observed is due to natural variation in the population.

2.2. Leaf cuticle wax extraction

To best represent a typical wax chemical composition for the ST and each type of variant, leaf samples were collected during the first year anthesis or the R4 stage of reproductive-floral development (Moore et al., 1991). Fom tillers having fully expanded second leaves below the flag leaf, leaf samples were collected in each of the three selected plants within a class. Leaf samples were excised and placed individually in pollination bags (No. 404, Lawson, Northfield IL) for transport to the lab and wax extraction (in less than 1 h). Mean wax amounts are presented in this report as the average of three selected plants representative of the ST and each of the variant types. In mid-leaf sections (22.5 × 1.3 cm (L × W)) including the mid-vein, leaf area was determined using a flatbed scanner and image analysis software (LIA32-ver.0.377e, Nagoya University, Nagoya, Japan). Leaf sections were cut into three segments, to fit inside 20 ml scintillation vials (Teflon-lined caps, VWR International, LLC, Brisbane CA), and submerged in 15 ml of hexane (99% GC hexane, Sigma-Aldrich LLC, St. Louis, MO) for 45 s before the wax extracts were dispensed into clean scintillation vials, as described by Jenks et al. (1995). The leaf segments were briefly (2 s) rinsed once more with new hexane (5 ml) and hexane fractions combined for each leaf sample. The samples appeared clear with a slight yellow tint (absent in green coloration typical of chlorophylls) before being evaporated to dryness.

2.3. Chemical analyses of waxes

Gas chromatography (GC) with flame ionization detection was performed using an Agilent 7890A GC-FID. The internal standard hexadecane (5 μg) was added to evaporated samples and waxes were derivatized by heating in (75 μl) *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA, Sigma-Aldrich LLC, St. Louis, MO) at 100 °C for 15 min. The BSTFA was evaporated under nitrogen and the samples were suspended in hexane (25 μl) for analyses. Compounds were injected in the GC and then separated using a 12 m, 0.2 μm HP-1 capillary column with helium as the carrier gas. The GC was programmed with an initial temperature of 80 °C and increased at 15 °C/min to 260 °C where the temperature was held for 10 min, then increased at 5 °C/min to 320 °C, where the temperature was held for 24 min for a total run time of 58 min. The composition of waxes were analyzed by a GC-Mass Spectrometer (MS) using an Agilent 7890A GC and 5975C Triple-Axis detector MS with 12 m, 0.2 μm HP-Ultra 1 capillary column with helium gas as a carrier, using a similar temperature profile as used for GC-FID. The molecular identities of individual wax compounds were determined by quadrupole electron impact GC–MS, using relative retention time and mass fragment spectra of each molecular species, in addition to comparisons to NIST MS Search 2.0 database and *bona fide* standards run on the same instrument. However, those missing from the library were compared to previously published spectra or elucidated from their ion fragmentation patterns (Tulloch and Hoffman, 1980; Wen and Jetter, 2009).

Quantification was based on FID peak areas, using Enhanced data analysis G1701EA software (Agilent Technologies, Santa Clara, CA), relative to the internal standard. Specific correction factors were developed from external standards and applied to the peak areas of the free fatty acids (C_{20}, C_{21}, C_{24}, C_{26}, and C_{28}), primary alcohols (C_{20}, C_{22}, C_{24}, C_{25}, C_{26}, C_{28}, C_{29}, and C_{30}) and alkanes (C_{23}, C_{24}, C_{25}, C_{26}, C_{27}, C_{28}, C_{29}, C_{30}, C_{31}, C_{32} and C_{33}). For all other peaks, a correction factor of 1 was assigned. A representative chromatogram of the 53 wax