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Proteome in regrowth cycles of sugarcane: Absence of proteins to tolerate adverse growth conditions may be related to reduced agricultural productivity

Gustavo Barizon Maranho[1], Rone Charles Maranho[1], Mariana Mancini Benez[1], Eduardo Jorge Pilau[2], Claudete Aparecida Mangolin[3] and Maria de Fátima Pires da Silva Machado[3]*

[1]Programa de Pós-graduação em Agronomia, Universidade Estadual de Maringá, Av. Colombo, 5790, 87020-900, Maringá, Paraná, Brazil. [2]Departamento de Química, Universidade Estadual de Maringá, Maringá, Paraná, Brazil. [3]Departamento de Biotecnologia, Genética e Biologia Celular, Universidade Estadual de Maringá, Maringá, Paraná, Brazil. *Author for correspondence. E-mail: mfpsmachado@uem.br

ABSTRACT. One of the main objectives of sugarcane plantations is to increase their longevity without decreasing agricultural productivity. In the present study, we analyzed the proteome of the axillary buds of 'RB966928' to investigate possible changes in the number of proteins at different cutting stages. Using tryptic digestion followed by ultra-performance liquid chromatography coupled with high-resolution time-of-flight mass spectrometry, 122 proteins were identified from the proteome of the axillary buds of 'RB966928'. Of the 122, respectively 97 and 95 proteins were detected at the first and fifth cutting stages, of which 27 and 25 proteins were unique to the respective stage. Proteins that prevent the misfolding of polypeptides generated under stress were exclusively detected at the first cutting stage. Meanwhile, proteins associated with stress responses and disease resistance were exclusively detected at the fifth cutting stage. The present proteomic analysis in the regrowth cycles and axillary bud development of 'RB966928' significantly advanced our understanding of the biological processes linked to the reduction of agricultural productivity of sugarcane with the advancement of cutting age. Absence of proteins to tolerate adverse growth conditions at the fifth cutting stage may be related to reduced agricultural productivity, in addition to environmental stress, soil compaction, nutrient availability, cultural practices, and pests or pathogen attacks at different phenological stages of crops.

Keywords: *Saccharum* spp.; longevity; productivity; stress proteins; differential proteome.

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Introduction

Increasing the longevity of sugarcane (*Saccharum* spp.) plantations without decreasing agricultural productivity is one of the main objectives of sugarcane cultivation. The productive cycle (longevity) of most sugarcane varieties in Brazil averages six years, with five cutting stages (Salomé, Sakai, & Ambrosano, 2007; Santos & Borém, 2013). Although it is common to find sugarcane at the fifth cutting stage, decrease in agricultural productivity is evident and intrinsic in each cultivar due to advanced cutting age (Dias, 2011; Barbosa et al., 2012). Moreover, this reduction in agricultural productivity may be related to environmental stresses, soil compaction, nutrient availability, cultural practices, and pest or pathogen attacks at different phenological stages of crops (Manhães, Garcia, Francelino, Francelino, & Coelho, 2015).

Changes in allele number and mean observed heterozygosity of the expressed sequence tag-simple sequence repeat (EST-SSR) loci at different (second, fourth and sixth) cutting stages have been detected in sugarcane cultivars 'RB72474' and 'RB867515' (Augusto, Maranho, Mangolin, Bespalhok Filho, and Machado, 2017). However, similarities and differences in 10 *Est-SSR* loci at the molecular level between 'RB72454' and 'RB867515' during subsequent cutting stages could not explain the frequently observed reduced productivity, but the differences reveal that phenotypic and physiological changes following each cutting stage were accompanied by changes at the genomic level. Biotic and abiotic factors may induce changes in gene expression, further decreasing the agricultural productivity of cultivars with high yield and wide adaptability ('RB72454') or high yield and sucrose content ('RB867515'), which are particularly important for ethanol and sugar production. Proteins and a set of proteins and polypeptides acting in response to different biotic and

abiotic factors may be found in the proteome of axillary buds of sugarcane. Sprouting of axillary buds is important to generate vigorous plants for the cultivated area and the interaction between genotype and environment should produce a proteome that allows the survival and continuity of production.

Proteomic analysis in sugarcane has been an useful tool for identification of proteins that responded to drought stress (Khueychai et al., 2015) salt stress (Passamani et al., 2017), infection by pathogen (Meng et al., 2020; Sánchez-Elordi et al., 2020; Zhou et al., 2021) and to identify proteins involved in cell wall biogenesis (Calderan-Rodrigues, Dantas, Gianotto, & Caldana, 2021) and proteins associated with sugarcane ratoon crop chlorosis (Fan et al., 2021). A reduction in the number of proteins in the axillary buds of 'RB867515' at the fifth cutting stage has been also demonstrated by Maranho et al. (2019). The above indicates reduction in the expression of genes that may be essential for the stability of the culture development.

Changes in gene expression of the axillary buds at the fifth cutting were detected in 'RB867515', and such changes may or not may be observed in other sugarcane cultivars. Since the establishment, maintenance, and expansion of a sugarcane farm depend on the budding potential of the axillary buds, the present study investigated whether reduction in the number of proteins from the first (plant cane) to the fifth (forth ratoon) cutting stage is an evidence that may be extended to other sugarcane cultivars important to the sugar and alcohol industries, such as 'RB966928'. 'RB966928' was developed through a cross between 'RB855156' and 'RB815690' and was released by the Federal University of Paraná in 2010. 'RB966928' is preferred by producers due to its qualities, such as high sucrose content at the beginning of the harvest; tall size; high tillering; eventual overturning; early maturation; high growth rate; indication for cultivation in a medium or under high-potential environments; and resistance to sugarcane smut (*Sporisorium scitamineum*), brown rust (*Puccinia melanocephala*), scald (*Xanthomonas albilineans*), and sugarcane mosaic virus (*Potyvirus* sp.). In addition, RB966928 stands out for its performance in both mechanized planting and harvesting (RIDESA, 2010; Daros, Oliveira, & Barbosa, 2015). In the 2017–2018 varietal census of the Instituto Agronômico de Campinas, 'RB966928' was the second most planted cultivar in the southern and central regions of Brazil (12.5% of total area) and the most planted cultivar for the renewal of sugarcane fields in the state of São Paulo, Brazil (Braga Junior et al., 2019).

To this end, we analyzed the proteome of the axillary buds of 'RB966928' to investigate the possible changes in the number of proteins at different cutting stages, with a reduction in this number from the first to the fifth cutting stage, as well as to catalog the protein products of differentially expressed genes at different cutting stages. A list of proteins altered after the first cutting stage in each sugarcane cultivar may be highly useful for selecting cultivars for crosses in breeding programs. Cultivars with agronomic characteristics of interest and negligible changes in proteins related to the metabolic pathways essential for their performance may be selected.

Material and methods

Sugarcane plants

Approximately 10-month-old 'RB966928' plants at the first and fifth cutting stages were collected from the Nova Aralco Industrial (20°53′29.82″ S, 50°26′55.25″ W) farm in the state of São Paulo, Brazil. The first and fifth cutting stages were selected since high productivity is generally observed at the first cutting stage, while marked decrease in the agricultural productivity is evident at the fifth cutting stage. The sugarcane plants were cultivated in Argisol Dystrophic soil with low water availability and moderate cation-exchange capacity. The sugarcane plants were collected in close sessions at least 10 m away from the edges of the field. Based on leaf count using the Kuijper's leaf numbering system, as described by Van Dillewijn (1952), axillary buds from the fourth to ninth node were used to avoid a greater influence of auxins in the axillary buds near the stem apex. Axillary buds of the canes at the first and fifth cutting stages were individually planted in vermiculite in labeled 10 L trays, with 3 cm spacing between the plants, to initiate sprouting. The plants were irrigated every second day.

Sprouting occurred in the greenhouse at 22°C after 5 days. The axillary buds of each cutting age (first and fifth stages) were cut with a scalpel, instantly frozen in liquid nitrogen, and stored in an ultra-freezer at -80°C until use. Eight axillary buds were selected from each plant. At each cutting age (first and fifth stages), the axillary buds of three plants (biological triplicate) were sampled and divided into three aliquots (technical triplicate).

Protein extraction, quantification, and digestion

Total proteins were extracted from the axillary buds (200 mg; eight axillary buds) of each plant using the modified TCA–acetone method for sugarcane (Maranho et al., 2018). Following extraction, the proteins were quantified by fluorimetry (Qubit Fluorometer 1.0; Invitrogen, Carlsbad, CA, USA) using the Qubit Protein Assay Kit (Invitrogen). The extracted proteins were pre-fractionated by one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (12% visualization gel and 5% stacking gel) for ~3 h at 200 V. The gel was then stained with 0.1% Coomassie Brilliant Blue R-250. SDS–PAGE verified the previously reported differences in protein expression in each sample and attested the protein extraction quality. The BenchMark Protein Ladder (10 to 220 KDa) was used to evaluate protein molecular weight.

Tryptic digestion followed the method described by Villén and Gygi (2008) with slight modifications. Disulfide bonds were digested by incubation in dithiothreitol (DTT) solution (5 mM DTT and 50 mM NH_4HCO_3). Cysteine residues were alkylated by incubation in an alkylation solution (14 mM iodoacetamide and 50 mM NH_4HCO_3). The proteins were digested by the Trypsin/Lys-C Mix (Promega, Madison, WI, USA) at a final concentration of 20 ng μL^{-1} for 16h at 37°C.

Ultra-performance liquid chromatography (UPLC), mass spectrometry (MS), and bioinformatics

The protein digest was analyzed by UPLC on the ACQUITY UPLC M-Class System (Waters, Milford, MA, USA) coupled to a high-resolution time-of-flight mass spectrometer (Xevo G2, Waters) equipped with an electrospray ionization source. Mass spectrometry-based proteomics has become the tool of choice for identifying and quantifying the proteome of organisms (Karpievitch, Polpitiya, Anderson, Smith, & Dabney, 2010). The exceptional sensitivity and resolving power of today's mass spectrometers allow for the detection of proteins and peptides at small amounts (Wither et al., 2016). Chromatographic separation was performed using the Acquity UPLC® M-Class HSS T3 Column (Waters, UK) (particle size, 1.8 µm; 300 µm × 150 mm) at a flow rate of 6 µL $min.^{-1}$. The gradient mixture of solvents A (H_2O with 0.1% formic acid; v:v) and B (acetonitrile with 0.1% formic acid; v/v) comprised 3% B 0-1 min., 40% B 1-80 min., 97% B 80-90 min., maintained at 97% B 90-97 min., 3% B 97-100 min., and maintained at 3% B 100-103 min. at 40°C. The capillary voltage was operated in the positive mode, at the following settings: 3.0 kV capillary voltage, 40 V sampling cone voltage, and 600 L h^{-1} desolvation gas at 400°C. Data were collected from m/z 50 to 2,000 via MSE acquisition; scan time was 0.5 X and ramp collision energy was 15-45 V.

Following UPLC-MS/MS, the data files (raw) were processed and analyzed using ProteinLynx Global ServerTM 3.0.3. Sequences were searched against the Viridiplantae and *Saccharum* taxonomy in the UniProtKB database (downloaded in April 2019). The following parameters were used for database searches: cleavage specificity: trypsin with 1 missed cleavage allowed; minimum fragment ion matches per peptide: 2; minimum fragment ion matches per protein: 5; minimum peptide matches per protein: 1; fixed modifier reagent: carbamidomethyl C; and variable modifier reagent: oxidation M.

Results and discussion

Bud sprouting

Five days after plantation in vermiculite, the sprouting rate of the axillary buds of 'RB966928' was higher at the first cutting stage (141/102, 72.3%) than at the fifth cutting stage (145/48, 26.9%).

'RB966928' proteome

The concentration of axillary bud proteins in the extract obtained using the modified TCA–acetone method was 747 µg mL^{-1} at the first cutting stage and 1.185 µg mL^{-1} at the fifth cutting stage.

SDS–PAGE showed differences in the protein expression profiles of the axillary buds between the first and fifth cutting stages (Figure 1). At both cutting stages, protein bands ranged between 120 and 25 kDa, and the regions with the highest protein amount and band intensity were 120 to 90 kDa, 60 to 50 kDa, 40 to 35 kDa, and 30 to 25 kDa.

Furthermore, 122 proteins were identified, of which respectively 97 and 95 were detected at the first and fifth cutting stages. Moreover, 27 (22.13% of the total proteome) (Table 1) and 25 (20.49% of the total proteome) (Table 2) proteins were exclusively detected at the first and fifth cutting stages, respectively, while 70 proteins were identified at both cutting stages (Figure 2).

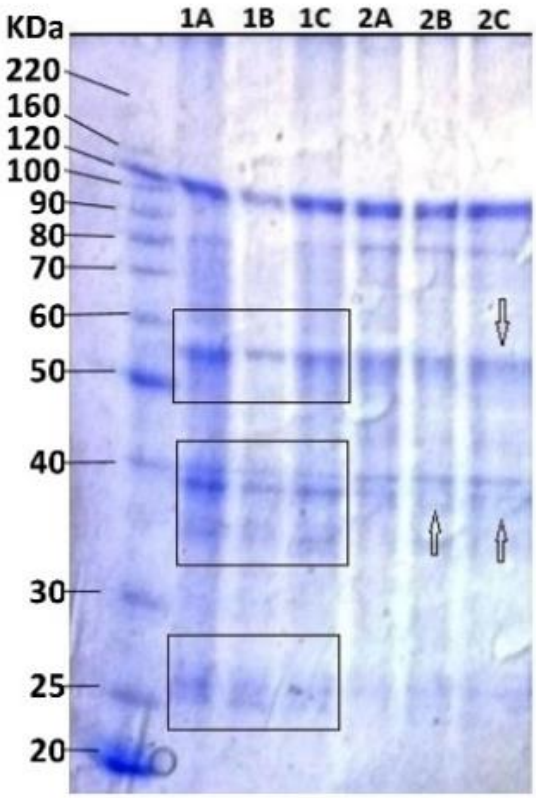

Figure 1. 1D SDS-PAGE with the protein profile from axillary buds of sugarcane cv. RB966928: black arrows (and rectangles) indicate differences between first and fifth cut. Lanes 1A– C, first cut; 2A-C, fifth cut; L, ladder.

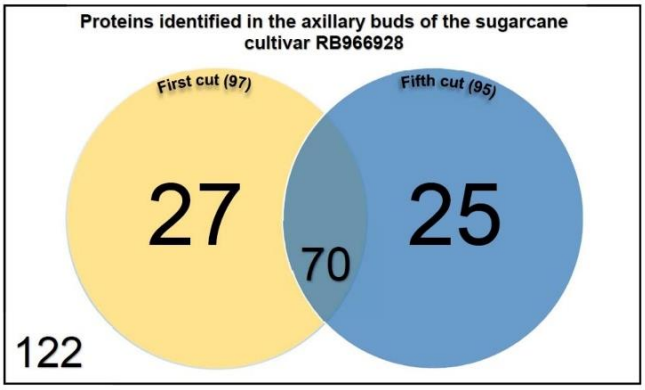

Figure 2. Venn diagram showing proteins identified in the axillary buds of the first cut (97) and the fifth cut (95) of the sugarcane cultivar RB966928, and proteins exclusively found in the first (27) and fifth (25) cutting.

The reduction in the number of proteins in the axillary buds was not significant between the first and fifth cutting stages of 'RB966928'. Accordingly, difference in the proteome of the axillary buds between the first and fifth cutting stages was smaller in 'RB966928' than in 'RB867515'. At the fifth cutting stage, the reduction in the number of proteins in the axillary buds was more remarkable in 'RB867515' than in 'RB966928' (Maranho et al., 2019). In addition, difference in the number of proteins in the axillary buds that were exclusively detected at the first and fifth cutting stages was smaller in 'RB966928' than in 'RB867515'. Thus, fewer proteins related to the metabolic pathways essential for performance were changed in 'RB966928' between the first and fifth cutting stages. Based on axillary bud gene expression, over 50% (62.5%) of the proteins were detected at both first and fifth cutting stages, whereas 46.4% were exclusively expressed at the first or fifth cutting stage.

The number of proteins detected in 'RB966928' in the present study was lower than that of protein detected in 'RB867515' in a previous study using 1DE-UPLC-ESI-Q-TOF (Maranho et al., 2019). Although 1DE-UPLC-ESI-Q-TOF detected more proteins, the proteomic analysis of 'RB966928' axillary buds with

tryptic digestion followed by UPLC coupled with high-resolution time-of-flight MS was faster, less labor intensive, and more cost-effective than the gel-based methodology adopted by Maranho et al. (2019).

Table 1. Proteins exclusively detected in axillary buds of the first cut (plant cane) of the sugarcane cv. RB966928.

No./Access or ID of proteins/ Database	Description	Score	No. of match	Relative mass (Da)	Protein Sequence Coverage (%)	Species
7/HS7E_SPIOL/P29357	Chloroplast envelope membrane 70 kDa heat shock-related protein	126.275.000	14	72.056	30.83	*Spinacia oleracea*
22/CH61_MAIZE/P29185	Chaperonin CPN60-1_ mitochondrial precursor (HSP60-1)	30.856.750	18	61.496	38.30	*Zea mays*
23/BIP3_MAIZE/O24581	Luminal binding protein 3 precursor (BiP3)	29.802.180	11	73.328	17.04	*Zea mays*
25/BIP5_TOBAC/Q03685	Luminal binding protein 5 precursor (BiP 5)	28.626.860	8	73.915	12.72	*Nicotiana tabacaum*
26/BIP4_TOBAC/Q03684	Luminal binding protein 4 precursor (BiP 4)	27.789.140	6	73.750	9.00	*Nicotiana tabacaum*
28/EF2_BETVU/O23755	Elongation factor 2 (EF-2)	27.146.780	15	94.768	22.66	*Beta vulgaris*
31/PGM2_MAIZE/P93805	Phosphoglucomutase_ cytoplasmic 2 (EC 5.4.2.2)	23.725.530	14	63.269	31.05	*Zea mays*
33/METE_SOLSC/Q42662	5-methyltetrahydropteroyltriglutamate-homocystein methyltransferase	21.941.780	10	84.875	14.27	*Plectranthus scutellarioides*
36/PAL1_ORYSA/P14717	Phenylalanine ammonia-lyase (EC 4.3.1.5)	20.462.080	12	76.379	17.26	*Oryza sativa* subsp. *japônica*
44/EF1D_ORYSA/P29545	Elongation factor 1-beta' (EF-1-beta')	15.613.790	6	23.698	34.23	*Oryza sativa* subsp. *japônica*
48/SUS2_MAIZE/P49036	Sucrose synthase 2 (EC 2.4.1.13)	13.601.710	14	93.566	18.14	*Zea mays*
56/PSA5_ORYSA/Q9LSU1	Proteasome subunit alpha type 5 (EC 3.4.25.1)	8.328.119	7	26.164	26.58	*Oryza sativa* subsp. *japônica*
68/RS15_ORYSA/P31674	40S ribosomal protein S15	4.641.314	1	17.381	12.50	*Oryza sativa* subsp. *japônica*
69/VATA_CITUN/Q9SM09	Vacuolar ATP synthase catalytic subunit A	4.589.085	8	68.966	17.50	*Citrus unshiu*
71/ACOC_CUCMA/P49608	Aconitate hydratase_ cytoplasmic (EC 4.2.1.3)	4.387.402	7	98.632	6.79	*Cucurbita máxima*
75/RUB2_BRANA/P34794	RuBisCO subunit binding-protein alpha subunit_ chloroplastic	3.897.729	4	61.719	7.38	*Brassica napus*
76/PS11_ARATH/P34066	Proteasome subunit alpha type 1-1 (EC 3.4.25.1)	3.762.358	2	30.704	4.32	*Arabidopsis thaliana*
79/CC48_SOYBN/P54774	Cell division cycle protein 48 homolog	2.843.157	10	90.568	12.52	*Glycine max*
82/MP13_AMBAR/P27761	Pollen allergen Amb a 1.3 precursor (Antigen E)	2.730.560	4	43.555	11.59	*Ambrosia artemisiifolia*
83/ODPB_PEA/P52904	Pyruvate dehydrogenase E1 component beta subunit	2.675.704	2	39.021	7.52	*Pisum sativum*
87/UBA1_WHEAT/P20973	Ubiquitin-activating enzyme E1 1	2.079.851	9	117.920	10.09	*Triticum aestivum*
89/AMP2_LYCES/Q42876	Aminopeptidase 2_ chloroplast precursor	2.037.322	2	60.119	4.22	*Solanum lycopersicum*
90/GLYM_SOLTU/P50433	Serine hydroxymethyltransferase_ mitochondrialF	2.005.779	5	57.260	9.46	*Solanum tuberosum*
91/PFPB_RICCO/Q41141	Pyrophosphate-fructose 6-phosphate 1-phosphotransferase subunit beta	1.953.333	4	60.684	2.72	*Ricinus communis*
92/PLD_MAIZE/Q43270	Phospholipase D precursor (EC 3.1.4.4) (PLD)	1.604.707	11	92.698	8.25	*Zea mays*
93/MHK_ARATH/P43294	Serine/threonine-protein kinase MHK (EC 2.7.1.-)	1.591.857	3	51.423	6.55	*Arabidopsis thaliana*
97/PURA_ARATH/Q96529	Adenylosuccinate synthetase_ chloroplast precursor	1.404.905	4	53.420	7.96	*Arabidopsis thaliana*

Table 2. Proteins exclusively detected in axillary buds of the fifth cut (forth ratoon) of the sugarcane cv. RB966928.

No./Access or ID of proteins/ Database	Description	Score	No. of match	Relative mass (Da)	Protein Sequence Coverage (%)	Species
14/GTH1_MAIZE/P12653	Glutathione S-transferase I (EC 2.5.1.18) (GST-I)	48.842.780	6	23.918	39.44	*Zea mays*
37/RAN2_ARATH/P41917	GTP-binding nuclear protein RAN-2	13.404.210	9	25.373	42.53	*Arabidopsis thaliana*
51/RS4_MAIZE/O22424	40S ribosomal protein S4	8.339.655	11	30.131	18.49	*Zea mays*
57/RL10_MAIZE/P45633	60S ribosomal protein L10 (QM protein homolog)	7.616.426	6	25.375	15.00	*Zea mays*
59/RS8_MAIZE/Q08069	40S ribosomal protein S8	7.571.969	7	25.170	23.08	*Zea mays*
60/RL2_ARATH/P46286	60S ribosomal protein L2	6.438.858	3	28.030	9.69	*Arabidopsis thaliana*
63/EFT2_SOYBN/P46280	Elongation factor Tu_ chloroplast precursor (EF-Tu)	6.004.597	13	52.623	16.49	*Glycine max*
65/SCRK_SOLTU/P37829	Fructokinase (EC 2.7.1.4)	5.380.151	5	33.992	6.27	*Solanum tuberosum*
67/RL12_PRUAR/O50003	60S ribosomal protein L12	5.317.468	6	17.996	13.25	*Prunus armeniaca*
68/143A_SOYBN/Q96450	14-3-3-like protein A (SGF14A)	5.195.375	9	29.162	26.46	*Glycine max*
71/1436_ARATH/P48349	14-3-3-like protein GF14 lambda	4.685.988	7	28.089	4.84	*Arabidopsis thaliana*
73/DMC1_LILLO/P37384	Meiotic recombination protein DMC1 homolog	4.418.811	6	38.557	20.92	*Lilium longiflorum*
74/1438_ARATH/P48348	14-3-3-like protein GF14 kappa	4.391.497	2	28.142	4.84	*Arabidopsis thaliana*
75/143A_LYCES/P93207	14-3-3-like protein 10 (Fragment)	4.285.310	1	15.841	8.70	*Solanum lycopersicum*
77/SPD1_ORYSA/Q9SMB1	Spermidine synthase 1 (EC 2.5.1.16)	4.171.835	4	35.545	14.86	*Oryza sativa* subsp. *japonica*
78/1434_LYCES/P42652	14-3-3-like protein 4 (PBLT4)	4.035.770	7	29.412	26.54	*Solanum lycopersicum*
79/GAPN_MAIZE/Q43272	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	3.963.740	15	53.773	28.71	*Zea mays*
80/1437_ARATH/Q96300	14-3-3-like protein GF14 nu	3.875.968	7	29.938	19.62	*Arabidopsis thaliana*
81/1435_ARATH/P42645	14-3-3-like protein GF14 upsilon	3.875.968	7	30.295	19.40	*Arabidopsis thaliana*
82/RL11_MEDSA/P46287	60S ribosomal protein L11 (L5)	3.858.915	3	20.854	11.60	*Medicago sativa*
84/PSA6_ORYSA/Q9LSU3	Proteasome subunit alpha type 6 (EC 3.4.25.1)	3.763.218	8	27.687	17.07	*Oryza sativa subsp. japonica*
86/NLT3_ORYSA/Q42999	Nonspecific lipid-transfer protein 3 precursor	3.639.888	3	12.094	35.90	*Oryza sativa subsp. japonica*
87/RUBB_SECCE/Q43831	RuBisCO subunit binding-protein beta subunit	3.608.982	14	53.754	15.23	*Secale cereal*
94/RBL_PETHY/P04992	Ribulose bisphosphate carboxylase large chain	2.619.578	2	53.489	12.16	*Petunia hybrid*
95/SMT3_ORYSA/P55857	Ubiquitin-like protein SMT3	2.451.094	2	11.042	29.00	*Oryza sativa* subsp. *japonica*

Analysis of the 122 proteins detected at the first and fifth cutting stages showed that these proteins were associated with several cellular and metabolic processes, such as carbohydrate, lipid, and protein metabolism or biotic and abiotic stress response. Ribosomal proteins, transcription and elongation factors, cellular (cytoplasmic and vacuolar) components, molecular chaperones, histones, and other important peptides for axillary bud development were identified.

Differential proteomes at the first and fifth cutting stages

The 25 proteins detected exclusively at the first cutting stage serve diverse functions in biological processes, and many of these proteins (and polypeptides) are involved in fundamental metabolic pathways of

plant development, including sprouting and budding. Five types of BiPs (BiP1, BiP2, Bip3, Bip4, and BiP5) were detected in the axillary buds of 'RB966928' at the first cutting stage, while only two BiPs (BiP1 and BiP2) were detected at the fifth cutting stage. BiPs are molecular chaperones of the endoplasmic reticulum (RE), which are involved in protein folding and maturation (Carvalho et al., 2014). In the absence of BiPs, many secretory pathway proteins do not assume their active conformation and precipitate in the RE (Pobre, Poet, & Hendershot, 2019). Several studies have demonstrated the roles of BiPs in plant protection under various stress conditions, such as in stress attenuation in RE (Costa et al., 2008), drought tolerance in soybean (*Glycine max*) and tobacco (*Nicotiana tabacum*) (Alvim et al., 2001; Valente et al., 2009), innate immune response (Wang, Weaver, Kesarwani, & Dong, 2005), and osmotic stress-induced cell death attenuation (Reis et al., 2011).

The chloroplast envelope membrane 70 kDa heat shock-related protein, chaperonin CPN60-1, mitochondrial precursor, cell division cycle protein 48 homolog, phenylalanine ammonia-lyase (PAL; EC 4.3.1.5), cytoplasmic phosphoglucomutase (cPGM; EC 5.4.2.2), sucrose synthase 2 (SuSy; EC 2.4.1.13), 5-methyltetrahydropteroyltriglutamate–homocysteine *S*-methyltransferase (MET6; 2.1.1.14), cytoplasmic aconitate hydratase (cACO; EC 4.2.1.3), pyruvate dehydrogenase E1 component beta subunit (PDH E1-β; EC 1.2.4.1), aminopeptidase 2 chloroplast precursor (LAPA2; EC 3.4.11.5), pyrophosphate-fructose 6-phosphate 1-phosphotransferase subunit beta (PFP-β; EC 2.7.1.90), phospholipase D precursor (PLD; EC 3.1.4.4), and serine–threonine kinase (MHK; EC 2.7.1) were also exclusively detected at the first cutting stage (Table 1).

Chaperonin CPN60-1, mitochondrial precursor, which was exclusively detected at the first cutting stage of 'RB966928', likely prevents the misfolding of polypeptides and promotes proper refolding and assembly of the unfolded polypeptides generated under stress conditions in the mitochondrial matrix. The chloroplast envelope membrane 70 kDa heat shock-related protein, which was also exclusively detected in the axillary buds of 'RB966928' at the first cutting stage, forms a complex of 11 heat-shock proteins (Hsps), which are induced under thermal stress. The Hsps are essential to protect cells from heat and various damages by normalizing cellular functions during recovery (Morimoto et al., 1994; Parsell & Lindquist, 1994; Nover et al., 1996). The exclusive detection of chloroplast envelope membrane 70 kDa heat shock-related protein and chaperonin CPN60-1, mitochondrial precursor in the axillary buds at the first cutting stage suggests that the absence of these proteins at the fifth cutting stage can lead to protein unfolding in the mitochondria and chloroplast, resulting in defects in photosynthesis and glycolysis.

The cell division cycle protein 48 homolog (a product of *CDC48*) is an essential protein for plant cell cycle progression and is a component of protein quality control in plant immunity against pathogen infection (Chisholm, Coaker, Day, & Staskawicz, 2006; Jones & Dangl, 2006; Dangl, Horvath, & Staskawicz, 2013). The *CDC48* gene serves essential regulatory functions during development and possibly contributes to protein degradation through the ubiquitin proteasome system (UPS) or ER-associated protein degradation. Plants utilize UPS to facilitate cellular changes required to respond to and tolerate adverse growth conditions (Stone, 2019). Exclusive detection of cell division cycle protein 48 homolog and ubiquitin-activating enzyme E1 1 in the axillary buds of 'RB966928' at the first stage suggests that defense responses to pathogens at the fourth ratoon may be weak, rendering the plants more vulnerable to pathogen attacks. Cell division cycle protein 48 homolog has been reported to regulate the turnover of immune receptors and act as a mediator of viral protein degradation (Bègue, Mounier, Rosnoblet, & Wendehenne, 2019).

Similarly, exclusive detection of PAL at the first cutting stage may render the fourth ratoon more susceptible to pathogen attack and external injuries to the plant stem inflicted by abiotic and biotic factors. The production of phytoalexins and phenylpropanoids following fungal infection involves rapid induction of PAL (Ritte & Schulz, 2004). In tobacco, the suppression of PAL protein expression resulted in various phenotypes, including reduced growth, altered leaf shape, reduced pollen viability, and increased susceptibility to the pathogenic fungus *Cercospora nicotianae* (Eichel, Gonzalez, Hotze, Matthews, & Schroder, 1990). Plants with low PAL activity have thinned cell walls and reduced lignin content in the secondary xylem, which provides mechanical stiffness and strength to the stem, allows upward growth, and enables water and mineral transport through the vascular tissue under negative pressure without tissue collapse.

Strongly reduced growth, characterized by decreased fresh weight, shortened roots, and reduced seed production, has been reported in *Arabidopsis* lacking cPGM (Malinova et al., 2014). In our study, cPGM was detected in the axillary buds at the first cutting stage but not at the fifth cutting stage of 'RB966928'. Thus, the absence of cPGM in the axillary buds of 'RB966928' at the fifth cutting stage may be associated with decreased agricultural productivity of sugarcane, with the plants becoming less robust and more susceptible to abiotic and biotic stresses with the advancement of cutting age.

Absence of other proteins including SuSy, MET6, cACO, LAPA2, PFP-β, PLD, and MHK in the axillary buds of 'RB966928' at the fifth cutting stage may reduce growth and decrease agricultural productivity of sugarcane at this stage. For instance, the products of sucrose cleavage by SuSy are available for many metabolic pathways, such as energy production, primary metabolite production, and complex carbohydrate synthesis. Plants with reduced SuSy activity have been shown to exhibit reduced growth, decreased starch content, limited cellulose or callose synthesis, reduced tolerance to anaerobic stress, and altered shoot apical meristem functions and leaf morphology. Meanwhile, plants overexpressing SuSy show augmented growth, increased xylem area and xylem cell wall thickness, and elevated cellulose and starch content, making SuSy a potent candidate for improving the agricultural traits of crops (reviewed by Stein & Granot, 2019).

MET6 catalyzes the last step in L-methionine biosynthesis (Eichel et al., 1995). Reduced aconitase activity led to a stunted phenotype at the early developmental stages of tomato (*Lycopersicon pennellii*) (Carrari et al., 2003). Absence of LAPA2 in the axillary buds at the fifth cutting stage may also affect plant development, since LAPA2 is presumably involved in the processing and regular turnover of intracellular proteins.

Furthermore, PFP-β and PLD were important enzymes exclusively detected at the first cutting stage. PFP-β catalyze the first committing step of glycolysis, while PLD catalyzes phospholipid hydrolysis to produce phosphatidic acid, which often serves as a secondary messenger in intracellular signal transduction (Kolesnikov et al., 2012). Absence of PFP-β may affect the equilibrium of carbon metabolism (Duan et al., 2016). MHK is implicated in biotic stress and hormonal response (Taj, Agarwal, Grant, & Kumar, 2010).

Furthermore, many proteins exclusively identified in the axillary buds of 'RB966928' at the fifth cutting stage are mainly linked to stress response and disease resistance. A series of proteins from the 14-3-3 (14-3-3-like family) protein family were exclusively detected at the fifth cutting stage. Seven 14-3-3 proteins were detected in the axillary buds at the fifth cutting stage, including 14-3-3-like protein A, 14-3-3-like protein GF14 lambda, 14-3-3-like protein GF14 kappa, 14-3-3-like protein 10, 14-3-3-like protein 4, 14-3-3-like protein GF14 nu, and 14-3-3-like protein GF14 upsilon. These seven 14-3-3-like family proteins, which were exclusively detected in the axillary buds of the fourth ratoon, are associated with the regulation of several important biochemical pathways and peroxide detoxification (Fulgosi et al., 2002). Maranho et al. (2019) detected 14-3-3-like proteins only in the fourth ratoon of 'RB867515'. Different genes encoding the 14-3-3 proteins have been identified in various plant species (Camoni, Iori, Marra, & Aducci, 2000). For instance, 15 isoforms of 14-3-3-like proteins have been detected in *Arabidopsis thaliana*, 14 of which are labeled using the Greek letters (Camoni et al., 2000; Denison, Paul, Zupanska, & Ferl, 2011). In recent years, increasing evidence of the involvement of 14-3-3 proteins in various aspects of plant hormonal physiology have been accumulated. The review by Camoni, Visconti, Aducci, and Marra (2018) offers novel insights into the roles of 14-3-3 proteins in the regulation of hormonal signaling, biosynthesis, and transport. To date, however, no study has evaluated the roles of 14-3-3 proteins in sugarcane axillary buds.

Furthermore, glutathione-*S*-transferases (GSTs; EC 2.5.1.18), spermidine synthases (SPDSs; EC 2.5.1.16), and meiotic recombination protein DMC1 homolog were exclusively expressed at the fifth cutting stage, which play roles in both normal cellular metabolism and stress response.

GSTs involved in normal cellular metabolism and detoxification of a wide variety of xenobiotics, including herbicides, has been reported in plants (Mannervik & Danielson, 1988). Early studies on the roles of GSTs in plant biotic stress showed that certain *GST* genes are specifically upregulated under microbial infection (Gullner, Komives, Király, & Schröder, 2018). Moreover, proteomic studies confirmed the accumulation of multiple GSTs in infected plants. The acknowledged roles of GSTs include detoxification through glutathione conjugation, oxidative stress relief, and hormone transport. Induction of the *GST* genes or elevation of the GST activity has often been observed in plants treated with beneficial microbes (bacteria and fungi), which induce a systemic resistance response to subsequent pathogen infections. A review by Kumar & Trivedi (2018) provides updated information on the roles of GSTs in abiotic and biotic stresses, with an emphasis on their uptake, metabolism, and detoxification in plants. In addition, increased GST9 levels have been reported with aging, suggesting a role related to senescence. Thus, GSTs, which were exclusively detected in the axillary buds of the fourth ratoon (fifth cutting stage), may reflect a response to field-applied herbicides and/or subsequent pathogen infections with the advancement of cutting age.

SPDS was another protein exclusively detected in the axillary buds of 'RB966928' at the fifth cutting stage, and it is associated with stress response. According to Kasukabe et al. (2004), spermidine plays important regulatory roles in stress signaling pathways, leading to increased stress tolerance in *Arabidopsis thaliana*.

SPDSs are dimeric enzymes that share the fold of polyamine biosynthetic proteins (Sekula & Dauter, 2019). Polyamines have been implicated in a wide range of biological processes during plant growth and development, including senescence, environmental stress response, and fungal or viral resistance (Sawhney, Tiburcio, Altabella, & Galston, 2003). Pál et al. (2018) detected high polyamine and proline levels under osmotic stress in wheat seedlings.

Meiotic recombination protein DMC1 homolog, also exclusively detected in the axillary buds of the fourth ratoon, has been identified as one of the two RecA homologs found in eukaryotic cells. DMC1 (disrupted meiotic cDNA) has been reported to play a central role in homologous recombination during meiosis through recruitment at the sites of programmed DNA double-strand breaks (DSBs). Szurman-Zubrzycka et al. (2019) reported that DMC1 is essential for DSB repair, crossing-over, and proper chromosome disjunction during meiosis in barley. Although DMC1 is predominantly involved in meiosis, efficient mediation of mitotic recombination by rad51 and Dmc1 in yeast cells has been reported (reviewed by Neale & Keeney, 2006). In our study, DMC1 was detected in the somatic cells of the axillary buds of 'RB966928'at the fifth cutting stage. Occurrence of somatic recombination in sugarcane has been proposed by Augusto et al. (2017) to explain the loss of alleles and/or reduction in heterozygous phenotypes by vegetative propagation following each cutting stage in 'RB72454' and 'RB867515'.

Overall, analysis of differential proteomes of the axillary buds of 'RB966928' at the first and fifth cutting stages revealed little changes in the number and composition of proteins essential for plant growth. The proteins exclusively detected at the first cutting stage prevent the misfolding of polypeptides and promote the proper refolding and assembly of the unfolded polypeptides generated under stress to tolerate adverse growth conditions. Furthermore, 40% of the proteins exclusively detected at fifth cutting stage are associated with stress responses and disease resistance. These results indicate that environmental stress, pathogen infection, and heavy metal contamination may reduce plant growth, rendering plants less robust and more susceptible to subsequent abiotic and biotic stresses and ultimately decreasing agricultural productivity of the sugarcane plantation with the advancement of cutting age.

Our findings suggest that if the expression of genes encoding proteins exclusive to the first cutting stage is induced at the fifth cutting stage, the decrease in bud sprouting and agricultural productivity of 'RB966928' with the advancement of cutting age may be attenuated. Listing of proteins that are altered from the first to fifth cutting stages in 'RB968628' may provide useful information for the selection of cultivars for crosses in breeding programs.

Conclusion

The present proteomic analysis in the regrowth cycles and axillary bud development of 'RB966928' significantly advanced our understanding of the biological processes linked to the reduction of agricultural productivity of sugarcane with the advancement of cutting age. Absence of proteins to tolerate adverse growth conditions at the fifth cutting stage may be related to reduced agricultural productivity, in addition to environmental stress, soil compaction, nutrient availability, cultural practices, and pests or pathogen attacks at different phenological stages of crops.

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