

Characterizing fruit ripening in plantain and Cavendish bananas: A proteomics approach



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ABSTRACT

The fruit physiology of banana cultivars other than Cavendish is poorly understood. To study the ripening process, samples were taken daily from plantain and Cavendish bananas and the ripening stages were determined. We present data from the green to the fully mature stage. By analyzing the protein abundances during ripening we provide some new insights into the ripening process and how plantains fruits are different. Multivariate analysis of the proteins was performed correlated to the starch dynamics. A drop in sucrose synthase and a rise of acid invertase during ripening indicated a change in the balance of the sucrose fate. During ripening, sugars may no longer be available for respiration since they are stored in the vacuoles, making citrate the preferred respiratory substrate. We found significant cultivar specific differences in granule-bound starch synthase, alpha- and beta amylases and cell wall invertase when comparing the protein content at the same ripening stage. This corroborates the difference in starch content/structure between both banana types. Differences in small heat shock proteins and in the cell wall-modifying enzyme xyloglucan endotransglucosylase/hydrolase support respectively the presumed higher carotenoid content and the firmer fruit structure of plantains.

Significance: We follow the proteome during ripening and correlate the proteins to the measured starch content. We discuss the changes in two contrasting genotypes. This gives us for the first time insight into the ripening of plantain and how this is different from the well-known Cavendish banana. This will revive the plantain breeding programs since for the first time we get insight into the plantain ripening.

1. Introduction

With 30,137,993 hg/ha, Banana (*Musa* spp.) was the tenth most important crop in terms of yield in 2017 (FOA Stat). It is produced in > 130 (sub)tropical countries across the globe. The most well-known banana is the export banana Cavendish. It contains relatively low amounts of starch and is eaten raw as a dessert fruit. However, the diversity of banana is huge and many banana varieties have higher starch content [1]. Depending on the variety, these starchy bananas have to be cooked (called cooking banana), fried or roasted. Plantain is a specific high starch containing banana variety that is cooked. Approximately 50% of the plantain production comes from the humid forest agro-ecological zone of West and Central Africa making it a very important crop for local farmers [2]. Plantains provide more than a quarter of the carbohydrate requirements for > 70 million people

making them one of the most important sources of food energy in West and Central Africa. Since it can guarantee a yield with little input, it has the potential to reduce hidden hunger.

The yield of plantain is affected by the susceptibility to diseases and pests, limited precipitation, low soil fertility, poor soil and crop management and the use of poor quality or disease-infected planting materials. Therefore, it is important to deliver improved varieties. Unfortunately, due to its sterility and its poor genetic and physiological characterization, the delivery of improved hybrids is very slow, hampering the breeding programs [3]. Although plantains are genetically similar [4], there are many phenotypic differences [5]. This phenotype is used to classify them in four subgroups; French, French Horn, False Horn and Horn [6,7].

A proteomic approach has paramount importance to study orphan species like banana [8]. Fruits vary between cultivars and the major

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physiological modifications that affect colour, texture, flavour, and aroma are under the control of both external (light and temperature) and internal (gene regulation and hormonal signaling) factors. Fruit ripening has different stages and involves several changes in the biochemistry, physiology and gene expression of the fruit. Differential proteomics at different stages of fruit ripening gives information on the concomitant molecular changes during this important developmental process [9,10]. Additionally, the identification of the proteins during ripening may provide hints for the regulation of metabolic pathways of fruits and should lead to identify proteins involved in various biological processes like carbohydrate metabolism, stress/defense, cell structure, secondary metabolism and energy. Proteomics (2 DE) has already contributed to the understanding of ripening in Cavendish banana [11]. Several ripening-associated genes change their expression during ripening in banana [12]. However, different banana cultivars contain different amounts of carbohydrate (starch) which convert into simple sugars at different rates (fructose, glucose and sucrose) [13]. The first proteome of plantain [14] did not present the dynamics of ripening. Since Cavendish fruits are soft and sweet at maturity and plantains starchy and harder, key proteins linked to major biochemical processes (starch metabolism) are presumed to be different. Such key proteins may regulate different metabolic pathways involved in a complex regulatory network and will help breeders in the future to understand the unique alleles in plantains. We present here the dynamics of the proteome in plantain and Cavendish during the different ripening stages and the correlation to the starch content and we discuss observed variety differences. Proteomics enhanced our understanding of the molecular basis of plantain fruit quality through the identification of proteins that are associated with fruit development and starch content.

2. Material and methods

2.1. Plant material

Cavendish (*Musa* AAA subgroup) and plantains (*Musa* AAB subgroup) fruits were bought at a local supermarket in Leuven, Belgium. Visual changes in fruit colour were observed by comparing the fruits to the ripening guide of the USDA. This colour chart shows the fruits and gives index numbers for the different stages: 1 (green), 2 (light green (breaking towards yellow)), 3 (yellowish), 4 (greenish (more yellow than green)), 5 (yellow with green tips), 6 (yellow), 7 (yellow, flecked with brown). All selected fruits were at stage 2 at purchase and were kept in the same incubator chamber at 30 °C to continue the ripening process.

2.2. Sample preparation and experimental design

Three biological replicates from both Cavendish and plantains fruits were collected from the incubator every day until they reached the senescence stage 7 (for Cavendish during 7 days and for plantain during 14 days). The peel was removed and the pulp was cut into small pieces and immediately transferred to liquid nitrogen to stop enzymatic activities. Samples were lyophilized using the lyophilizer Christ alpha 1–4 LSC, and lyophilizer Christ alpha 1–4 LSCplus, ANALIS SA/NV. The same fruit was used for protein and starch analysis.

2.3. Starch quantification

The quantification of starch was done with the starch assay kit (Sigma-Aldrich, USA; Catalog Number SA20) following the manufacturer advice.

2.4. Proteomics

2.4.1. Extraction of proteins

All chemical were of analytical grade or higher. Extractions were

performed using the phenol extraction/ammonium acetate precipitation described for bananas by Carpentier et al. [15] and adapted by Buts et al. [16]. Briefly, 150 mg of lyophilized sample was extracted with ice-cold Tris buffered phenol (pH 8.0) (Acros). Samples were kept at 4 °C at all time and were protected with protease inhibitor cocktail (Roche Applied Science). The phenolic phase was washed and the proteins were precipitated with 5 volumes of 100 mM ammonium acetate in methanol (VWR). The pellet was washed with acetone, dried, resuspended in 100 µL of 8 M Urea (GE healthcare), 30 mM Tris, 5 mM DTT (GE healthcare) and quantified (GE healthcare 2D Quant Kit). Twenty µg of proteins were reduced and alkylated and digested overnight at 37 °C in 50 mM ammonium bicarbonate (Fluka) and 0.2 µg of trypsin (Pierce MS grade, Thermo Scientific). The peptide samples were desalted using Pierce C18 spin columns (Thermo Scientific) according to the manufacturer's instructions.

2.4.2. LC-MS/MS

The digested samples (1 µg) were injected (5 µL) and separated on an Ultimate 3000 UPLC system (Dionex, Thermo Scientific) equipped with an Acclaim PepMap100 pre-column (C18 3 µm–100 Å, Thermo Scientific) and a C18 PepMap RSLC (2 µm, 50 µm–15 cm, Thermo Scientific) using a linear gradient (300 µL/min) of 0–4% buffer B (80% ACN, 0.08% FA) in 3 min, 4–10% B in 12 min, 10–35% in 20 min, 35–65% in 5 min, 65–95% in 1 min 95% for 10 min, 95–5% in 1 min, 5% 10 min. The Q Exactive Orbitrap mass spectrometer (Thermo Scientific) was operated in positive ion mode with a nanospray voltage of 1.5 kV and a source temperature of 250 °C. Proteo Mass LTQ/FT-Hybrid ESI Pos Mode Cal Mix (MS CAL5-1EASUPELCO, Sigma-Aldrich) was used as an external calibrant and the lock mass 445.12003 as an internal calibrant. The instrument was operated in data-dependent acquisition (DDA) mode with a survey MS scan at a resolution of 70,000 (FWHM at m/z 200) for the mass range of m/z 400–1600 for precursor ions, followed by MS/MS scans of the top ten most intense peaks with +2, +3, +4, and +5 charged ions above a threshold ion count of 16,000 at 17500 resolution using normalized collision energy (NCE) of 25 eV with an isolation window of 3.0 m/z and dynamic exclusion of 10 s. All data were acquired with Xcalibur 3.0.63 software (Thermo Scientific).

2.4.3. Protein quantification and function analysis

For identification, all data were converted into mgf. files by the software Progenesis version 4.1 and processed using MASCOT version 2.2.06 (Matrix Science) against our in-house *Musa* A_B database (76,220 accessions). The parameters used to search at MASCOT were: parent tolerance of 10 PPM, fragment tolerance of 20 mmu, variable modification oxidation of M, fixed modification with carbamidomethyl C and up to one missed cleavage for trypsin. To calculate the FDR [17] and judge the protein inference, the sample-specific mgf files were loaded into Scaffold 3.6.5. The proteins were quantified in Progenesis based on all matching features. For the annotation of the proteins, the accessions were blasted against the banana genome and annotations were inferred from uniprot (trembl + swissprot) [18]. Protein-annotation relations were visualized in cytoscape [19] (See Supplementary cytoscape files) and proteins with shared Biological Process annotations were manually checked and grouped in the results tables. The GreenPhylV4 (www.greenphylo.org), UniProtKB (<https://www.uniprot.org/>), the banana hub (<https://banana-genome-hub.southgreen.fr/>) and the KEGG Pathway (<http://www.genome.jp/kegg/pathway.html>) platforms were used for data interpretation.

2.5. Statistical analysis

Protein quantifications were exported from Progenesis and subjected for additional statistical analysis (Statistica 13, Tibco). For proteomics analysis, the ANOVA value generated by Progenesis was corrected for repeated testing [20] and the significant proteins ($p \leq .01$)

Table 1

Biological process grouping of the overall significant correlations between proteins and starch for both cultivars together.

Accession number	Correlation to starch	Function	Number of peptides used for quantification
Glycolytic process GO:0006096			
GSMUA_Achr10T29150_001	0,64	2-3-bisphosphoglycerate-independent phosphoglycerate mutase	13
KMMuB_chr6_G15889	0,60	Fructokinase 1	6
GSMUA_Achr6T25020_001	0,59	Sulfite reductase [ferredoxin]	8
KMMuB_chr2_G04637	0,57	Pyruvate kinase	14
GSMUA_Achr9T15740_001	0,55	Putative 1-aminocyclopropane-1-carboxylate deaminase	1
GSMUA_AchrUn_randomT11210_001	0,45	Malate dehydrogenase, mitochondrial	11
KMMuB_chr1_G01610	-0,46	Pyruvate dehydrogenase e1 component subunit alpha- mitochondrial-like	11
KMMuB_chr5_G14647	-0,48	Pyruvate decarboxylase	4
GSMUA_Achr5T28140_001	-0,48	Pyruvate decarboxylase isozyme 2	6
GSMUA_Achr9T30280_001	-0,63	Putative aconitate hydratase	19
GSMUA_Achr8T22810_001	-0,70	Glutamate decarboxylase 1	13
KMMuB_chr8_G24037	-0,70	Glutamate decarboxylase	13
GSMUA_Achr11T01170_001	-0,76	Aconitate hydratase 2, mitochondrial	23
KMMuB_chr11_G31784	-0,82	Aconitate cytoplasmic-like	23
Carbohydrate metabolic process GO:0005975			
GSMUA_Achr5T08130_001	0,54	Alpha-amylase isozyme 3C	6
GSMUA_Achr5T08040_001	-0,49	Putative Inactive beta-amylase 9	6
GSMUA_Achr11T06330_001	-0,53	Putative Alpha-glucosidase 2	9
GSMUA_AchrUn_randomT06130_001	-0,66	Probable xyloglucan endotransglucosylase/hydrolase protein 32	9
GSMUA_Achr3T14470_001	-0,64	Alpha-glucan water dikinase, chloroplastic	37
KMMuB_chr3_G06616	-0,68	Alpha-glucan water dikinase chloroplastic-like	30
GSMUA_Achr8T04140_001	-0,68	Alpha-amylase	9
Protein folding GO:0006457			
GSMUA_Achr6T16580_001	0,67	Peptidyl-prolyl cis-trans isomerase	6
GSMUA_Achr10T26930_001	0,63	Putative Heat shock protein 90	6
GSMUA_Achr2T12540_001	0,57	Small heat shock protein	4
KMMuB_chr3_G06296	0,56	Cyclophilin	13
GSMUA_AchrUn_randomT08730_001	0,53	RuBisCO large subunit-binding protein subunit alpha	14
GSMUA_Achr8T21710_001	0,53	10 kDa chaperonin	6
KMMuB_chr1_G01211	0,52	Chaperone protein 1-like	8
GSMUA_Achr11T1160_001	0,52	Chaperonin CPN60-2	16
KMMuB_chr1_G01860	0,45	kda class i heat shock protein	13
GSMUA_Achr11T03720_001	-0,60	Putative Heat shock protein STI	7
Translation GO:0006412			
KMMuB_chr11_G33846	0,69	Elongation factor 1-	8
GSMUA_Achr2T06640_001	0,64	Elongation factor 1-delta	6
GSMUA_Achr10T12900_001	-0,54	60S ribosomal protein L10	5
Transport GO:0006810			
KMMuB_chr1_G00260	0,63	Monosaccharide-sensing protein 2-like	5
KMMuB_chrUn_random_G37150	0,57	Atpase subunit 1	17
KMMuB_chr4_G10064	0,56	Lipid transfer protein	2
GSMUA_Achr8T14200_001	0,56	Mitochondrial outer membrane protein porin of 36 kDa	2
GSMUA_Achr7T23900_001	0,48	Mitochondrial outer membrane protein porin	6
GSMUA_Achr4T04300_001	-0,52	Coatomer subunit delta-2	4
Cysteine biosynthetic process GO:0019344			
GSMUA_Achr6T16580_001	0,67	Peptidyl-prolyl cis-trans isomerase	6
GSMUA_Achr6T25020_001	0,59	Sulfite reductase [ferredoxin]	8
KMMuB_chrUn_random_G37293	0,58	Aspartyl protease	6
Methylation GO:0032259			
GSMUA_Achr3T14020_001	0,59	Regulator of ribonuclease-like protein 2	4
KMMuB_chr7_G18687	0,49	5-methyltetrahydropteroyl-triglutamate-homocysteine expressed	19
KMMuB_chr1_G00130	-0,68	8-hydroxyquercetin 8-o-methyltransferase	7
Oxidation-reduction process GO:0055114			
GSMUA_Achr10T00490_001	0,68	Tropinone reductase homolog At1g07440	5
KMMuB_chr1_G00260	0,63	Monosaccharide-sensing protein 2-like	5
GSMUA_Achr6T25020_001	0,59	Sulfite reductase [ferredoxin]	8
KMMuB_chr3_G05265	0,51	Formate dehydrogenase	22
KMMuB_chr10_G30370	0,47	Mitochondrial protoporphyrinogen oxidase	4
GSMUA_Achr10T15890_001	0,47	Putative Peroxidase 12	2
KMMuB_chr1_G01610	-0,46	Pyruvate dehydrogenase e1 component subunit alpha- mitochondrial-like	11
GSMUA_Achr11T08370_001	-0,51	Ferritin-3	3
KMMuB_chr1_G00487	-0,52	nadp-specific isocitrate	14
KMMuB_chr10_G30931	-0,56	Lysine-ketoglutarate reductase saccharopine dehydrogenase bifunctional enzyme	9
GSMUA_Achr11T23780_001	-0,6	Ferritin-3	5
Negative regulation of catalytic activity GO:0043086			
GSMUA_Achr3T14020_001	0,59	Regulator of ribonuclease-like protein 2	4
GSMUA_Achr3T05740_001	-0,48	Pectinesterase/pectinesterase inhibitor PPE8B	8

(continued on next page)

Table 1 (continued)

Accession number	Correlation to starch	Function	Number of peptides used for quantification
KMMuB_chr3_G05670	-0,50	Pectinesterase	10
GSMUA_Achr1T15410_001	-0,68	Putative invertase inhibitor	5
One-carbon metabolic process GO:0006730			
KMMuB_chr10_G31494	0,72	10-formyltetrahydrofolate synthetase	6
KMMuB_chr9_G25379	0,60	Digalactosyldiacylglycerol synthase chloroplastic-like	5
Metabolic process GO:0008152			
GSMUA_Achr9T04210_001	0,71	Protein IN2-1 homolog B	4
GSMUA_Achr2T02330_001	0,69	Probable glutathione S-transferase GSTF1	5
KMMuB_chr7_G19852	0,60	3 -n-debenzoyl-2 -deoxytaxol n-benzoyltransferase	6
GSMUA_Achr3T12590_001	0,57	Probable inactive purple acid phosphatase 29	4
GSMUA_Achr4T01500_001	0,56	Thioredoxin-like protein Clot	2
GSMUA_Achr11T20440_001	0,56	Glutathione S-transferase 3	3
KMMuB_chr6_G18191	0,56	in2-1 protein	4
GSMUA_Achr5T05290_001	0,54	Aldehyde dehydrogenase family 2 member B7, mitochondrial	19
KMMuB_chr10_G30212	0,50	gds1 esterase lipase	4
GSMUA_Achr1T15360_001	0,49	3-ketoacyl-CoA thiolase 2, peroxisomal	2
GSMUA_Achr2T11130_001	0,46	Putative GDSL esterase/lipase At5g55050	5
KMMuB_chr1_G02258	-0,50	3 -n-debenzoyl-2 -deoxytaxol n-benzoyltransferase-like	4
KMMuB_chr6_G16263	-0,57	Acetolactate synthase	11
GSMUA_AchrUn_randomT09150_001	-0,57	Putative peroxisomal-coenzyme A synthetase	7
KMMuB_chr6_G17898	-0,58	Probable carboxylesterase 15-like	5
GSMUA_Achr1T20250_001	-0,58	Putative 3'-N-debenzoyl-2'-deoxytaxol N-benzoyltransferase	8
GSMUA_Achr2T03950_001	-0,69	Putative peroxisomal-coenzyme A synthetase	14
No shared BP annotation			
GSMUA_Achr2T03470_001	0,69	Proteasome subunit alpha type-7	5
GSMUA_Achr9T30930_001	0,64	Acyl carrier protein 1, chloroplastic	4
GSMUA_Achr5T01150_001	0,62	Subtilisin N-terminal Region family protein, expressed	5
KMMuB_chr8_G21831	0,62	gdp-mannose 3 -epimerase	19
GSMUA_Achr7T07190_001	0,61	Hypothetical protein	3
GSMUA_Achr11T20310_001	0,61	Elongation factor 1-beta 1	6
GSMUA_Achr8T04450_001	0,60	GDP-mannose 3,5-epimerase 2	18
KMMuB_chr7_G18804	0,60	Selenium binding protein	3
GSMUA_Achr6T08230_001	0,59	DAG protein, chloroplast precursor, putative, expressed	2
KMMuB_chr1_G01051	0,57	Guanine nucleotide-binding protein subunit beta-like protein	9
KMMuB_chr8_G24976	0,57	Acyl carrier protein 1	2
GSMUA_Achr6T31470_001	0,57	Thaumatococcus-like protein	7
KMMuB_chr6_G18040	0,55	Thaumatococcus-like protein	15
GSMUA_Achr6T33160_001	0,55	Protein IN2-1 homolog B	6
GSMUA_Achr5T16020_001	0,55	Auxin-induced protein PCNT115	7
KMMuB_chr7_G19042	0,53	Methylthioribose kinase	7
KMMuB_chr10_G30049	0,53	udp-sulfoquinovose synthase	1
KMMuB_chr11_G32949	0,52	Mannose-binding lectin	5
KMMuB_chr11_G34247	0,51	r40g2 protein	8
KMMuB_chr2_G04758	0,50	appr-1-p processing enzyme family protein	2
GSMUA_Achr11T19070_001	0,50	Putative Basic leucine zipper and W2 domain-containing protein 2	5
GSMUA_Achr6T16180_001	0,49	Putative Diphosphomevalonate decarboxylase	4
KMMuB_chr5_G12234	0,48	Protein	15
GSMUA_Achr8T24060_001	0,48	Putative Dihydroflavonol-4-reductase	2
GSMUA_Achr7T01530_001	0,48	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	19
KMMuB_chr10_G30906	0,47	Legumin-like protein	4
GSMUA_Achr2T19390_001	0,46	Profilin	3
KMMuB_chr8_G21749	0,46	Cysteine protease 1 precursor	5
KMMuB_chr9_G26619	0,46	Os08g0455800	1
GSMUA_Achr3T23430_001	0,45	Putative 3-mercaptopyruvate sulfurtransferase	3
GSMUA_Achr11T14050_001	0,45	Dihydrolipoyl dehydrogenase 1, mitochondrial	9
GSMUA_Achr10T23170_001	-0,46	Alpha-aminoacidic semialdehyde synthase	8
GSMUA_Achr10T06760_001	-0,47	Serine carboxypeptidase-like 51	4
GSMUA_Achr4T22360_001	-0,49	Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial	6
GSMUA_Achr9T07800_001	-0,52	Cytochrome b-c1 complex subunit 7	2
GSMUA_Achr4T15090_001	-0,53	Expressed protein	4
GSMUA_Achr1T25050_001	-0,53	Putative 3'-N-debenzoyl-2'-deoxytaxol N-benzoyltransferase	5
GSMUA_Achr1T05110_001	-0,54	Isocitrate dehydrogenase [NADP]	17
KMMuB_chr4_G08808	-0,63	Glutelin type-a 1-like	7
KMMuB_chr1_G00939	-0,64	Autoinhibited h + atpase	6
GSMUA_Achr4T16020_001	-0,65	Putative Levodione reductase	9
GSMUA_Achr4T04730_001	-0,65	Putative cupin domain containing protein, expressed	9
GSMUA_Achr4T16570_001	-0,69	Putative O-methyltransferase ZRP4	8

were submitted for 2 way ANOVA (Table 2) using Statistica (Statistica 13, Tibco). Protein abundances were investigated in relation to the starch content via the Partial Least Squares-Discriminant Analysis PLS-

DA (Statistica 13, Tibco NIPALS algorithm, starch content as dependent variable and protein abundances as continuous predictors). Variable importance pointed towards possible significant correlations. The top

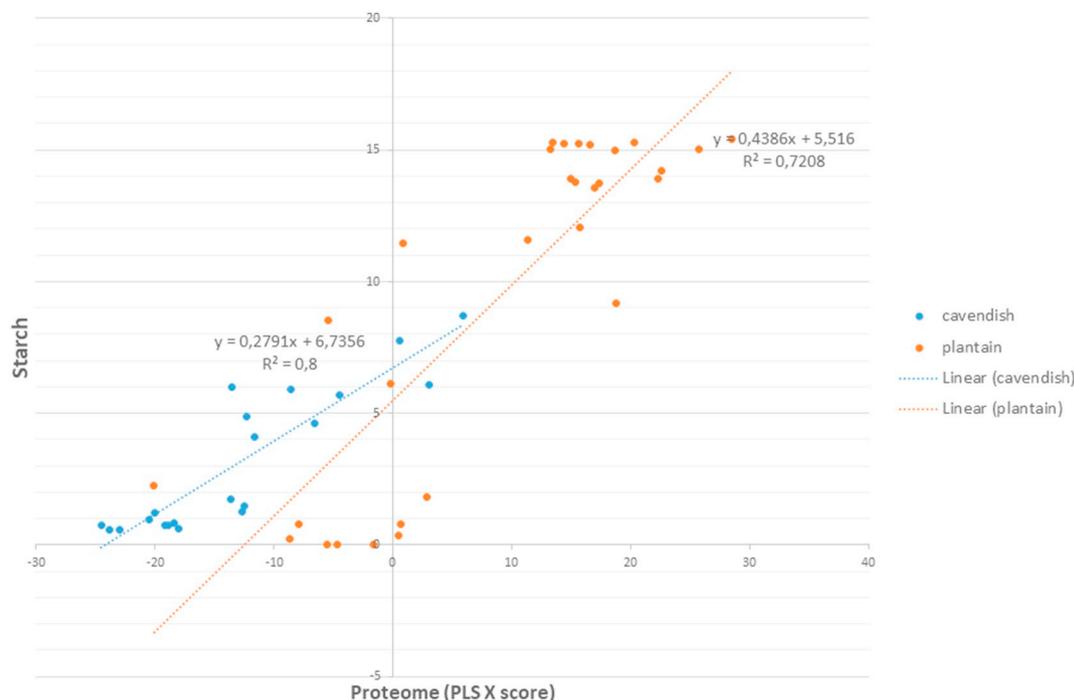


Fig. 1. Correlation between the proteome and the starch content of a fruit. The first component of the PLS explains 77% of sum of squares of the dependent variable (starch).

100 most important proteins were subjected to multiple regression analysis (Statistica 13, Tibco). Only correlations between protein and starch content that have a p value $\leq .001$ have been retained (Table 1).

3. Results and discussion

3.1. Proteome dynamics during ripening

We investigated the differences in protein abundance with the progression of starch breakdown to understand key proteins involved in the fruit ripening in both Cavendish and plantain. Therefore, LC-MS/MS-based proteomic analysis was conducted. We have identified 1021 proteins with a protein FDR of 0.6%. The list of non-redundant proteins and the presumed annotation of the proteins are presented in Supplementary Table 1. A PLS-DA analysis where correlations are calculated between the proteome of Cavendish and plantain and their corresponding starch content shows that the overall correlation between the proteome and the starch content is high and significant (R^2 0.77) with a slightly higher correlation in Cavendish than in plantain (R^2 resp. 0.8 and 0.72, Fig. 1). Table 1 shows the overall correlation between proteins and starch content for both varieties together. We only considered correlations between protein and starch content that have a P value $\leq .001$. Table 2 shows the proteins that were significantly different when subjected to 2 way ANOVA. The abundance and the dynamics of each protein, are displayed in supplementary file 1. Below we describe the most relevant proteins related to the changes during the ripening process based on the significant ANOVA score and/or significant correlation.

3.2. Starch metabolism

3.2.1. Alpha-glucan water dikinase

The degradation of starch is primed by the addition of phosphate groups catalyzed by the enzyme glucan water dikinase to disrupt the packing of the glucans at the granule surface [21]. The phosphate groups may influence the packing of the glucose polymers within the granule and hence the susceptibility of the granule surface to be

degraded by enzymes [22]. We found an excellent overall correlation to starch (-0.68 and -0.64 resp. for KMMuB_chr3_G06616 / GSMUA_Achr3T14470_001, Table 1). There is also a significant difference for KMMuB_chr3_G06616 for the ripening stage (Table 2, Supplementary file 1).

3.2.2. α amylase

We have identified 1 α amylase isoform GSMUA_Achr8T04140_001 as having an excellent overall correlation to starch (-0.68) (Table 1). This same isoform GSMUA_Achr8T04140_001 was also confirmed to be upregulated during ripening in other studies [12,23]. Our analysis confirms the one of Gao and coworkers [23], showing that α amylase plays a major role in starch degradation in both genotypes.

Moreover, in plantain, there is an excellent and significant positive correlation between the alpha-amylase GSMUA_Achr8T04140_001 and glucan water dikinase KMMuB_chr3_G06616: 0.84 (Fig. 2). This excellent mutual correlation and the high negative correlation to starch shows that when the starch content is high, the protein abundances are low. This indicates that both enzymes are more important in plantain near the end of the starch breakdown process when the starch is probably depolymerized and much simpler in structure [22].

We also identified an alpha-amylase like isozyme GSMUA_Achr5T08130_001 that had a significant positive overall correlation to the starch content (0.54) (Table 1) but it is significantly higher abundant in plantain (Table 2, Supplementary file 1). The higher levels of this alpha-amylase in plantain might be related to the higher starch content and/or the different composition. It is known that there is a variation in starch granule structure in plantains compared to Cavendish. Plantains contain more starch, a higher ratio resistant-starch/total starch and smaller and more ellipsoidal granules compared to Cavendish [23,24].

3.2.3. Beta-amylase

A negative correlation between beta-amylase and starch was found by Nascimento and coworkers [25]. In our analysis, GSMUA_Achr5T08040_001 had indeed an overall high negative correlation to starch (-0.49 Table 1) indicating it has an important role to

Table 2
Biological process grouping of the significant proteins according to 2 way ANOVA.

^a	Accession	Description	Peptides used for quantitation	p value for ripening stage ^b	p value for ripening stage*genotype ^b	P value genotype ^b
Carbohydrate metabolic process GO:0005975						
3	KMMuB_chr11_G31784	Aconitate cytoplasmic-like	23	0,00	0,19	0,00
12	GSMUA_Achr5T08130_001	Alpha-amylase isozyme 3C	6	0,36	0,32	0,00
13	GSMUA_Achr2T21870_001	Sucrose synthase 2	12	0,00	0,00	0,82
17	GSMUA_Achr1T08190_001	Granule-bound starch synthase 1, chloroplastic/amyloplastic	34	0,02	0,00	0,00
18	KMMuB_chr3_G05330	Sucrose synthase	14	0,00	0,00	0,60
22	KMMuB_chr8_G24908	Acidic endochitinase	4	0,01	0,02	0,00
23	KMMuB_chr5_G14647	Pyruvate decarboxylase	4	0,00	0,00	0,00
27	GSMUA_Achr5T08040_001	Putative Inactive beta-amylase 9	6	0,34	0,04	0,00
28	GSMUA_AchrUn_randomG06130_001	Probable xyloglucan endotransglucosylase/hydrolase protein 32	9	0,00	0,66	0,00
31	GSMUA_AchrUn_randomG24840_001	Beta-fructofuranosidase, insoluble isoenzyme 3	5	0,00	0,01	0,00
32	KMMuB_chr6_G15948	Cell wall invertase	7	0,00	0,02	0,00
34	KMMuB_chr6_G17733	gdsI esterase lipase at3g26430-like	6	0,74	0,43	0,00
39	KMMuB_chr6_G16263	Acetolactate synthase	11	0,02	0,58	0,00
40	KMMuB_chr3_G06616	Alpha-glucan water dikinase chloroplastic-like	30	0,01	0,07	0,14
44	GSMUA_Achr8T07170_001	Obg-like ATPase 1	3	0,19	0,37	0,00
45	GSMUA_Achr6T12500_001	Fructokinase-2	2	0,52	0,67	0,00
Oxidation-reduction process GO:005114						
4	GSMUA_Achr10T00490_001	Tropinone reductase homolog At1g07440	5	0,09	0,08	0,00
11	GSMUA_Achr10T15890_001	Putative Peroxidase 12	2	0,08	0,08	0,00
30	KMMuB_chr7_G20627	1-aminocyclopropane-1-carboxylate oxidase	15	0,83	0,88	0,00
35	KMMuB_chr6_G17844	Polyphenol oxidase	13	0,20	0,01	0,00
36	GSMUA_Achr6T29370_001	Polyphenol oxidase, chloroplastic	13	0,38	0,02	0,00
Metabolic process GO:0008152						
2	KMMuB_chrUn_random_G35118	Probable pectate lyase 15-like	8	0,01	0,01	0,00
5	GSMUA_AchrUn_randomG04250_001	Probable pectate lyase 15	6	0,01	0,03	0,00
21	GSMUA_AchrUn_randomG01190_001	Ankyrin repeat domain-containing protein 2	5	0,00	0,00	0,08
29	GSMUA_Achr2T02330_001	Probable glutathione S-transferase GSTF1	5	0,01	0,27	0,00
33	GSMUA_Achr9T04210_001	Protein IN2-1 homolog B	4	0,19	0,39	0,00
38	KMMuB_chr6_G17898	Probable carboxylesterase 15-like	5	0,15	0,66	0,00
Transport GO:0006810						
25	KMMuB_chr8_G24300	Adenine nucleotide translocator	11	0,94	0,77	0,00
26	KMMuB_chr3_G06229	Carrier protein mitochondrial-like	11	0,94	0,77	0,00
Protein folding GO:0006457						
1	GSMUA_Achr2T12540_001	Small heat shock protein, chloroplastic	4	1,00	0,98	0,00
6	KMMuB_chr1_G01860	kda class i heat shock protein	13	0,31	0,30	0,00
7	GSMUA_Achr1T21160_001	18.5 kDa class I heat shock protein	12	0,35	0,34	0,00
8	KMMuB_chr9_G25107	Chaperone	10	0,78	0,74	0,00
10	KMMuB_chr9_G25108	Chaperone	7	0,83	0,77	0,00
24	GSMUA_Achr11T03720_001	Putative Heat shock protein STI	7	0,00	0,02	0,00
42	KMMuB_chr8_G21678	kda class i heat shock protein	5	0,78	0,58	0,00
No shared BP annotation						
14	KMMuB_chr8_G24976	Acyl carrier protein 1	2	0,21	0,21	0,00
15	KMMuB_chr7_G18804	Selenium binding protein	3	0,92	0,90	0,00
16	KMMuB_chr1_G00130	8-hydroxyquercetin 8-o-methyltransferase	7	0,00	0,07	0,00
19	GSMUA_Achr7T13650_001	Mitochondrial-processing peptidase subunit alpha	7	0,43	0,28	0,00
20	GSMUA_Achr4T16570_001	Putative O-methyltransferase ZRP4	8	0,00	0,11	0,00
37	GSMUA_Achr10T09210_001	Putative Subtilisin-like protease	1	0,05	0,05	0,01
41	KMMuB_chr10_G30049	udp-sulfoquinovose synthase	1	0,11	0,82	0,57
43	KMMuB_chr9_G26109	Polygalacturonase-inhibiting protein	5	0,02	0,26	0,00
46	GSMUA_Achr9T12430_001	Putative Polygalacturonase inhibitor	7	0,22	0,52	0,00
9	GSMUA_Achr7T07190_001	Hypothetical protein	3	0,05	0,01	0,00

^a Refers to the slide number in the supplementary file 1.

^b All ANOVA overall values are corrected for repeated testing ($p \leq .01$).

play in starch breakdown in both genotypes. Gao and coworkers affirmed, based on qRT-PCR, that beta-amylase plays indeed a central role in starch breakdown but it did not explain the differences in the starch breakdown between Cavendish and plantain [23]. This does not completely match with our data at the protein level. GSMUA_Achr5T08040_001 is significantly more abundant in Cavendish

and highly abundant from the beginning (Table 2, Supplementary file 1). Beta-amylase is presumed to catalyze primarily linear glucan chains [22]. The starch structure in plantain is therefore presumably still too complex during initial ripening for beta amylase to act. The observed increase in abundance of beta-amylase at later ripening in plantain could be optimal for breakdown once the starch is more simple. In

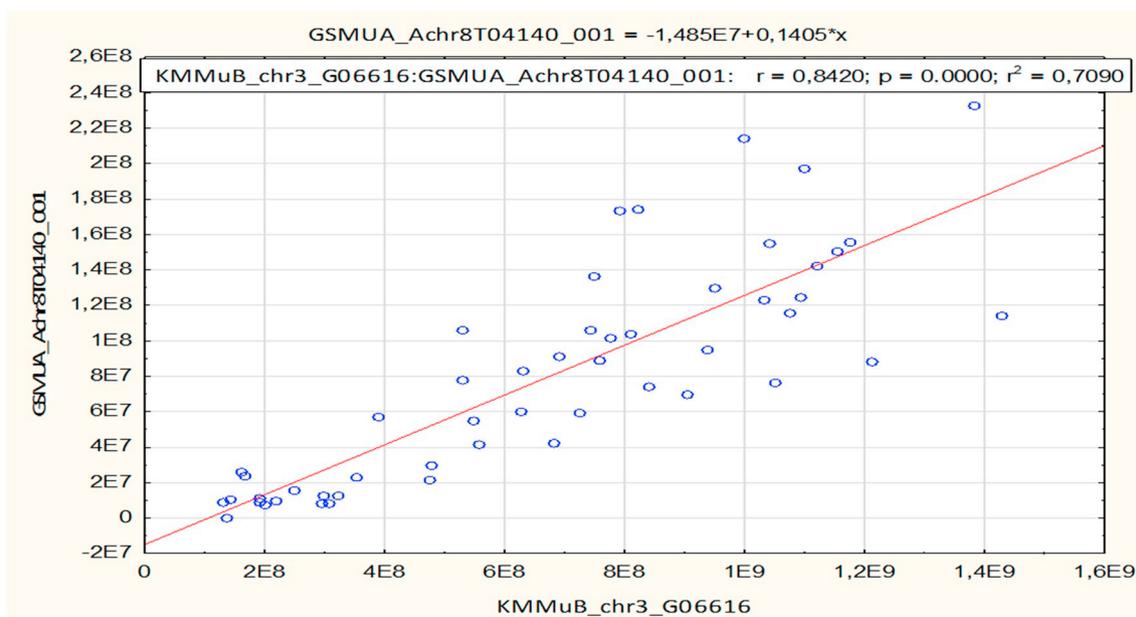


Fig. 2. Correlation between alpha-amylase (GSMUA_Achr8T04140_001) and glucan water dikinase (KMMuB_chr3_G06616).

Cavendish, beta amylase might already be able to break down the starch from the early ripening stages onwards. It has been reported that the amount of resistant starch is much higher in plantains compared to Cavendish [23]. To confirm our hypothesis, more studies are needed to check the difference in starch formation during the earlier stages of fruit development.

3.2.4. alpha-glucan phosphorylase

Toledo and coworkers identified in Cavendish via cross-species annotation, an alpha-glucan phosphorylase as an important enzyme that is popping up during the climacteric phase [11]. Starch phosphorylase (EC 2.4.1.1) catalyzes the reversible conversion of alpha-1,4-glucan and Pi into glucose-1-phosphate. Toledo and coworkers have identified an up till then unidentified isoform that contributes to the ethylene-responsive increase in starch breakdown since the activity of the enzyme seems to be regulated by the level of protein synthesis [11]. A blast of their protein hits GSMUA_Achr6T33840_001 and GSMUA_Achr4T32600_001. We have identified both proteins and found no significant correlation with starch and no significant differences between the 2 types of bananas. This indicates that this enzyme is not rate limiting during the starch breakdown. This would mean that ethylene switches on the production of this enzyme and then the enzyme level stays high during the whole starch breakdown process.

3.2.5. Granule-bound starch synthase

It is widely accepted that granule-bound starch synthases synthesize amylose whereas the soluble synthases catalyze distinct, but partly overlapping steps in the amylopectin biosynthesis [26]. We identified granule-bound starch synthase (GSMUA_Achr1T08190_001). It was significantly higher abundant in Cavendish and there was a significant genotype ripening interaction, meaning that both genotypes did not respond in the same way (Table 2, Supplementary file 1). In Cavendish granule bound starch synthase declines during ripening (Supplementary file 1). This decline during ripening has been reported in literature [27]. The higher abundance in Cavendish might also explain the difference in starch structure that has been reported [23]. The high abundance of granule-bound starch synthase points towards the fact that amylose synthesis still takes place in harvested Cavendish bananas. Since the fruits were already harvested, the building bricks for starch synthesis cannot come from source tissues. The building bricks for starch synthesis in a harvested banana could only come from the

soluble sugars produced during starch breakdown [12]. This points towards the existence of a cycle where starch is being broken down and stored as sucrose that is then broken down by sucrose synthase (see sucrose metabolism below) and resynthesized into amylose via granule-bound starch synthase. The fact that granule-bound starch synthase and sucrose synthase (see sucrose metabolism further) both steeply decrease during ripening indicates a shift in the balance between starch synthesis and starch breakdown. This shift in balance towards a higher breakdown of starch is probably caused by the fact that at a certain moment the sugars produced from starch breakdown are no longer returning to starch synthesis but are stored in the vacuole.

3.3. Sucrose metabolism

3.3.1. Sucrose synthase

Sucrose synthase activity in banana fruit in general, decreases drastically at the onset of fruit ripening and it is almost abolished during ripening on the plant (in-planta), or after harvest of green fruit and treatment by ethylene (ex-planta) [28,29]. Our results confirm this affirmations (Table 2, Fig. 3). We have identified several proteins as sucrose synthase: KMMuB_chr3_G05330/GSMUA_Achr3T1900_001, GSMUA_Achr2T21870_001, GSMUA_Achr8T22470_001/ KMMuB_chr8_G24007. All of them are positively correlated with starch so the lower the starch content the lower the sucrose synthase abundance (Table 1). Other researchers concluded that sucrose synthase activity is important for starch formation, but not for sucrose-synthesis related to fruit ripening [28,29]. Sucrose synthase has also been proposed to produce ADP-glucose linked to starch biosynthesis [30]. Based on all these studies and on our results, we conclude that sucrose synthase acts as the dominant enzyme in developing fruits to break down sucrose received from the source tissues and starch breakdown and contributes to the cycle of starch (re)formation (see also above granule-bound starch synthases). The significant decrease in both banana types, points towards a major shift in the starch cycle and drives the cycle towards final starch breakdown.

3.3.2. Sucrose phosphate synthase

Sucrose phosphate synthase is considered as the determinant enzyme responsible for sucrose synthesis during ripening. We identified 2 isoforms of SPS GSMUA_Achr4T16070_001 and GSMUA_Achr9T22510_001 (Supplementary Table 1). None of them are significantly different nor

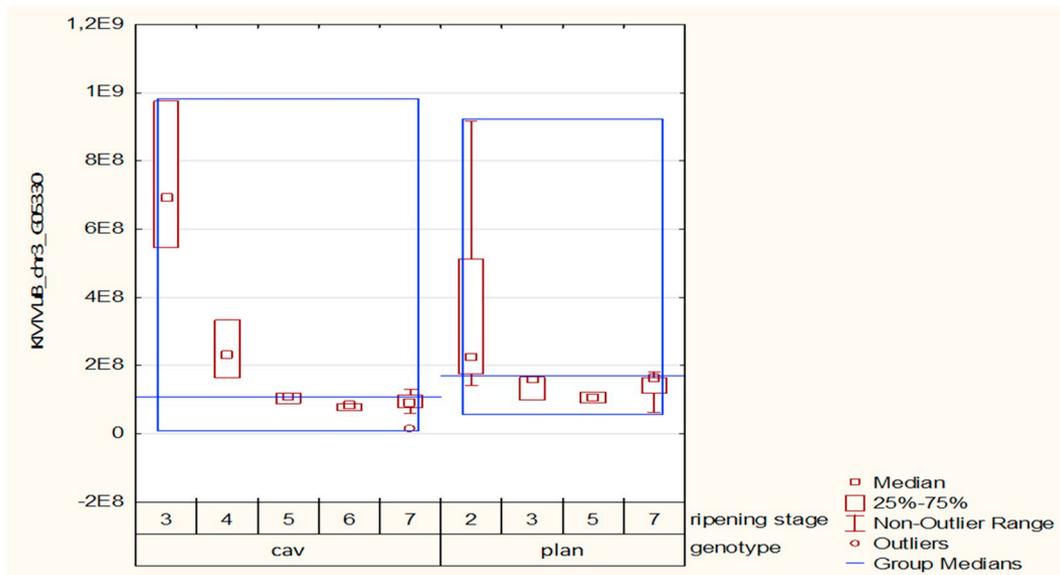


Fig. 3. Sucrose synthase abundance in both genotypes during the different ripening stages. The number of fruits analyzed per ripening stage (N) varies between 3 and 15 (N = 3–15).

correlated to starch. This was also observed by Fils-Lycaon [31].

3.3.3. Invertase

The sucrose formed from starch breakdown at the late phases of fruit ripening is broken down by invertase and not by sucrose synthase. Invertase activity of plantain has been reported to increase during post-harvest ripening [32]. We have identified acidic cell wall invertase isoforms KMMuB_chr6_G15948/GSMUA_Achr6T13160_001 and KMMuB_chrUn_random_G39074 /GSMUA_AchrUn_randomT24840_001. They increase in abundance with the ripening stage in both banana types but they are more abundant in plantain (Table 2, Fig. 4). Fils-Lycaon and coworkers reported that plantain decreased its sucrose/glucose + fructose ratio rapidly when reaching the fully ripe stage [31]. This corresponds with our observation that invertase is the main sucrose catabolizing enzyme at this stage. The fact that plantain showed higher invertase abundances than Cavendish might lead to a lower sucrose/glucose + fructose ratio. Fils-Lycaon and coworkers presented 2 cooking banana varieties that had a very low sucrose/glucose + fructose ratio with a sucrose level close

to zero in the final stages of ripening [31]. They discovered that those varieties had more than a 6-fold higher acid invertase activity. The observed 4.4 difference in mean invertase abundances between Cavendish and plantain might be responsible for the difference in taste and might be related to a different sucrose/glucose + fructose ratio.

3.4. Proteins associated with cell wall metabolism and softening

Fruit softening is one of the major observed phenotypic change during ripening. In tomato, this process involves disassembly of polysaccharide-rich cell walls, a reduction in cell-to-cell adhesion and changes in cuticle properties. Here we discuss some proteins that we identified and are related to cell wall metabolism.

3.4.1. Xyloglucan endotransglucosylase/hydrolase

Activation of different sets of cell wall-modifying enzymes plays an important role during fruit ripening. Xyloglucan endotransglucosylase/hydrolase (GSMUA_AchrUn_randomT06130_001) participates in fruit

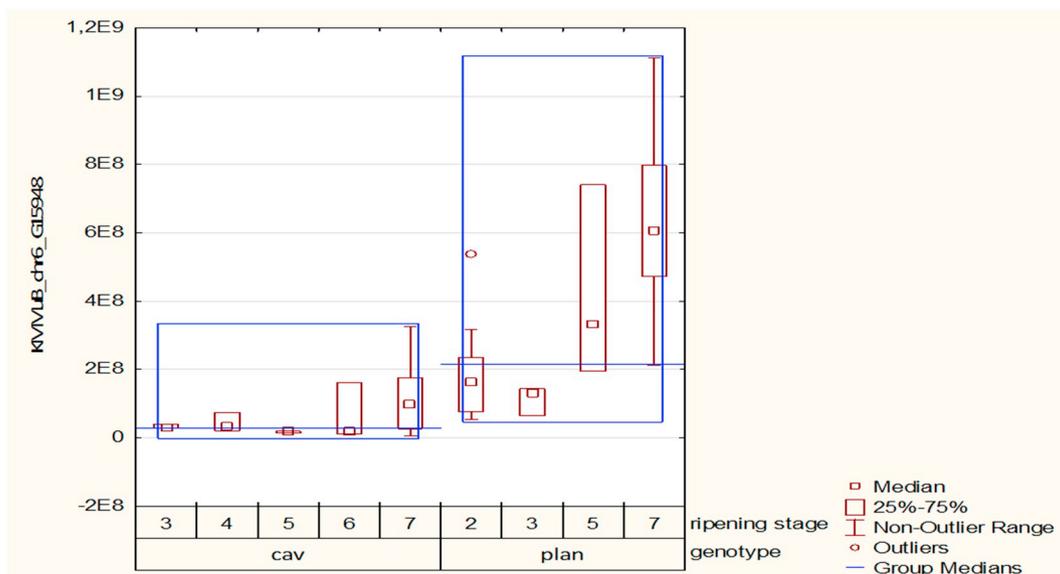


Fig. 4. Invertase abundance in both genotypes during the different ripening stages. (N = 3–15).

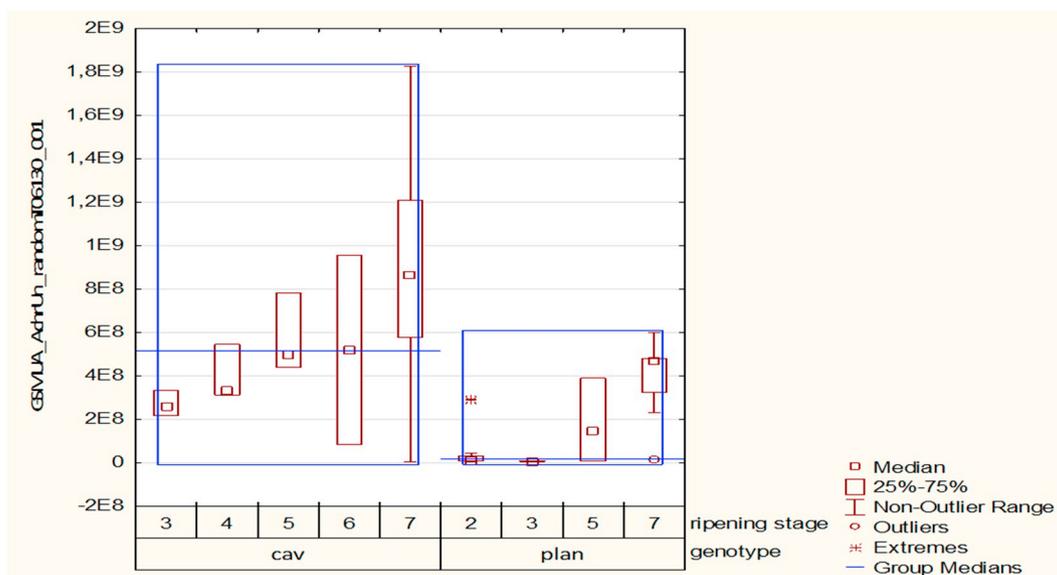


Fig. 5. Xyloglucan endotransglucosylase/hydrolase abundance during the different ripening stages in Cavendish and plantain. (N = 3–15).

softening [33]. We found a significant difference between Cavendish and plantain, which might explain the difference in softness at full ripening (Fig. 5; Table 2). At full ripening, Cavendish is a sweet tasting soft fruit that is easily eaten raw as a dessert while plantain is firmer than banana [34,35].

3.4.2. Pectin

We identified pectase lyase enzymes that were significantly higher in Cavendish (KMMuB_chrUn_random_G35118, GSMUA_AchrUn_randomT04250_001) (Table 2, Supplementary file 1). Silencing pectate lyase resulted in tomato in changes in fruit firmness with no obvious effects on either yield or weight, ethylene biosynthesis, colour or total soluble solids [36]. In our work the differential abundance of this enzyme might explain the difference in firmness between Cavendish and plantain. Additionally, a pectinesterase was found significantly negatively correlated to starch GSMUA_Achr3T05740_001/KMMuB_chr3_G05670 (Table 1).

3.4.3. Polyphenol oxidase

Polyphenol oxidase isoforms have been identified and were significantly more abundant in Cavendish:GSMUA_Achr6T29370_001/KMMuB_chr6_G17844,GSMUA_AchrUn_randomT22740_001, (Table 2, Supplementary file 1). This enzyme is present in most of the higher plants and its activity has an essential role in the enzymatic browning of raw fruits and vegetables. This phenomenon is an important factor impacting fruit quality since it brings undesirable effects in colour, taste, flavour and nutritional value. In the literature it is found that PPO activity increases during the maturation process. In medlar fruits, it was shown that PPO activity gradually decreases during fruit development followed by an increase during the maturation process [37]. In tomato, a PPO isoform showed a constitutively activity in different tissues and was developmentally regulated and ethylene induced [38]. In apple, the intensity between the polyphenol content and PPO activity is genotype-specific [39]. The fact that we found highly abundant PPO in Cavendish from the beginning and significantly more abundant than in plantain could be explained by the upregulation during ripening and seems to be genotype specific as in apple. This might make Cavendish more vulnerable to browning than plantain. More studies are necessary to confirm this.

3.4.4. Small heat shock proteins

Several small heat shock protein (sHSPs) isoforms have been

identified and were significantly more abundant in plantain (GSMUA_Achr2T12540_001, GSMUA_Achr1T21160_001/KMMuB_chr1_G01860, KMMuB_chr8_G21678; KMMuB_chr9_G25107; KMMuB_chr9_G25108) (Table 2, Supplementary file 1). In tomato transcripts were first detected at the turning stage when carotenoids start to accumulate in the fruits and are correlated to the accumulation of carotenoids during ripening [40]. The presence of HSP may be required for carotenoid accumulation. The same observation was made in strawberry fruits [41]. These results suggest a role for sHSPs in fruit development and specifically carotenoid formation. Our findings corroborate with this theory. It was shown that plantains contain higher carotenoid contents than Cavendish types [42]. This fact can explain the higher abundance of sHSPs in plantains.

3.5. Respiration and activation of the GABA shunt

We identified aconitase (KMMuB_chr11_G31784/GSMUA_Achr11T01170_001 and GSMUA_Achr9T30280_001), the enzyme that metabolizes citrate, and all showed a good correlation with starch (Table 1). It increases in abundance in both genotypes during ripening, though it is significantly higher abundant in Cavendish (Table 2, Fig. 6). During ripening, sugars may no longer be available for respiration since they are stored in the vacuoles, making organic acids (especially citrate) the preferred respiratory substrate [43]. The higher abundance in Cavendish might point towards a bigger deficiency of sugars as respiratory source. Aconitase was already reported in literature to be upregulated during ripening [27]. Plantains have a lower pH than Cavendish [35]. This might be related to the higher abundance of aconitase.

Malic acid and citric acid are the two main organic acids in banana [44]. Once citrate has been produced by the TCA cycle, it can be degraded in the cytosol [43]. One way to metabolize citric acid is the γ - aminobutyric acid (GABA) shunt. The GABA shunt leads to succinate and glutamine synthesis and is responsible for a decrease in fruit acidity [43]. Glutamate conversion to GABA through glutamate decarboxylase consumes protons mediating an increase in pH. Activation of the GABA shunt appears to occur during post-harvest ripening of banana since an accumulation of 2-Oxoglutarate has been shown [45].

The upregulation of aconitase was also observed before [27]. Cytosolic aconitase catalyzes the reversible conversion of citrate into isocitrate and it is known to be involved in the catabolism of citrate through the GABA shunt. The strong negative correlation of cytosolic

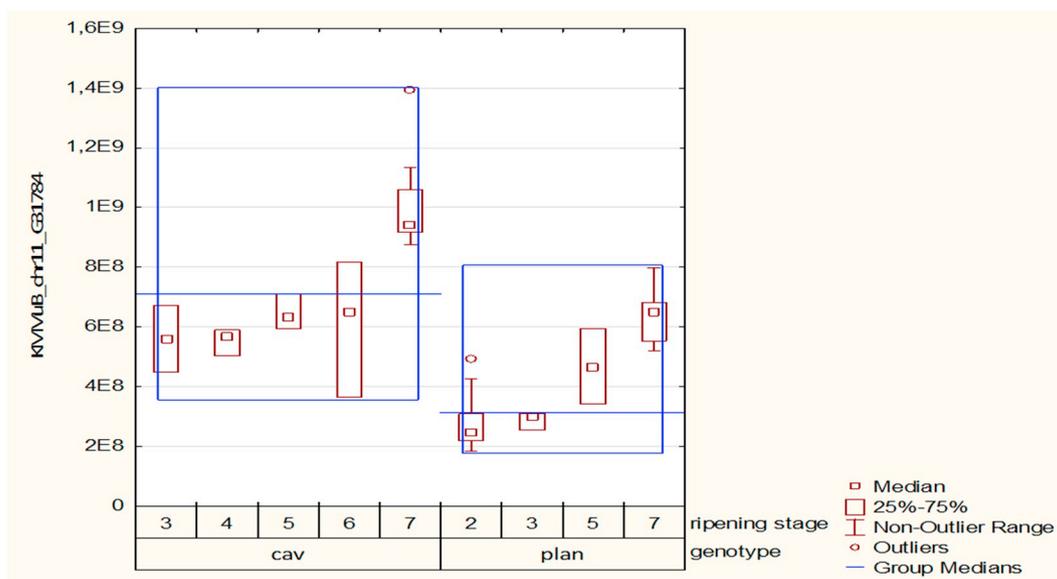


Fig. 6. Aconitase abundance during the different ripening stages in Cavendish and plantain. (N = 3–15).

aconitase to the starch content (Table 1) makes us hypothesize that it is likely that the rate of citrate degradation through the GABA shunt is in banana mainly controlled by cytosolic aconitase. Also, the next step in the shunt, the conversion from isocitrate into 2-oxoglutarate mediated by isocitrate dehydrogenase, is negatively correlated to starch (GSMUA_Achr1T05110_001/ KMMuB_chr1_G00487, Table 1). The last step of the GABA shunt, the glutamate decarboxylases GSMUA_Achr8T22810_001/ KMMuB_chr8_G24037, is similarly negatively correlated to starch (Table 1).

4. Conclusion

From literature it is known that plantains are bananas with a firm pulp texture, a high starch content and a high carotenoid content and Cavendish is a sweet dessert banana with a soft pulp texture and a low carotenoid content. By analyzing the enzyme abundances during ripening and correlating this to the starch content, we provide insights into the dynamics of the banana ripening process, how plantains are different and link it to their different phenotype. We found significant cultivar specific differences in the abundance of proteins involved in starch formation and degradation, sugar metabolism, cell wall degradation, polyphenol oxidation-reduction and the GABA shunt.

Associated data

The data have been deposited in PRIDE “Data are available via ProteomeXchange with identifier PXD013724.

Declaration of Competing Interest

The authors have declared no conflict of interest.

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<http://www.cgiar.org/who-we-are/>), and in particular to the CGIAR Research Program on Roots, Tubers, and Bananas.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jprot.2019.103632>.

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