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ORIGINAL ARTICLE

An overview of the transcriptional responses of two tolerant and susceptible sugarcane cultivars to borer (*Diatraea saccharalis*) infestation

Uilian Stefanello de Mello[1] · Pedro Marcus Pereira Vidigal[2] · Camilo Elber Vital[3] · Adriano Cirino Tomaz[1] · Milene de Figueiredo[1] · Luiz Alexandre Peternelli[4] · Márcio Henrique Pereira Barbosa[1]

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Abstract

Diatraea saccharalis constitutes a threat to the sugarcane productivity, and obtaining borer tolerant cultivars is an alternative method of control. Although there are studies about the relationship between the interaction of *D. saccharalis* with sugarcane, little is known about the molecular and genomic basis of defense mechanisms that confer tolerance to sugarcane cultivars. Here, we analyzed the transcriptional profile of two sugarcane cultivars in response to borer attack, RB867515 and SP80-3280, which are considered tolerant and sensitive to the borer attack, respectively. A sugarcane genome and transcriptome were used for read mapping. Differentially expressed transcripts and genes were identified and termed to as DETs and DEGs, according to the sugarcane database adopted. A total of 745 DETs and 416 DEGs were identified ($\log_2|\text{ratio}| > 0.81$; FDR corrected P value ≤ 0.01) after borer infestation. Following annotation of up- and down-regulated DETs and DEGs by similarity searches, the sugarcane cultivars demonstrated an up-regulation of jasmonic acid (JA), ethylene (ET), and defense protein genes, as well as a down-regulation of pathways involved in photosynthesis and energy metabolism. The expression analysis also highlighted that RB867515 cultivar is possibly more transcriptionally activated after 12 h from infestation than SP80-3280, which could imply in quicker responses by probably triggering more defense-related genes and mediating metabolic pathways to cope with borer attack.

Keywords Sugarcane · Borer · RNA-seq · Transcriptional response · Defense · Tolerance.

Introduction

A threat to sugarcane productivity is the presence of pests which cause economic losses in the field. The sugarcane borer (*Diatraea saccharalis*) is the main pest in Brazilian sugarcane growing areas. Estimates have demonstrated that each 1% of sugarcane internode infested with borer results in 0.5 to 1.1% of sugar yield loss (White et al. 2008; Rossato et al. 2013). Sugarcane breeders have focused on the selection of tolerant cultivars through conventional breeding methods aiming to control this pest and reduce its impact. However, studies have been focusing on the phenotypic characterization of sugarcane tolerance to borer (Dinardo-Miranda et al. 2012; Tomaz et al. 2017), and until now, little is known about genes that are involved in resistance mechanisms to this pest in sugarcane.

In general, the plant's defense mechanisms against insect-feeding involve the recognition of insect elicitors, followed by induction of a cascade of signal transduction pathways which leads to transcriptional changes of defense-related genes and

Uilian Stefanello de Mello and Pedro Marcus Pereira Vidigal contributed equally to this work.

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✉ Pedro Marcus Pereira Vidigal
pedro.vidigal@ufv.br

[1] Department of Agronomy, Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais, Brazil

[2] Núcleo de Análise de Biomoléculas (NuBioMol), Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais, Brazil

[3] Department of Biochemistry and Molecular Biology, Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais, Brazil

[4] Department of Statistics, Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais, Brazil

consequently triggering the biosynthesis of defense metabolites. Throughout evolution, plants have been acquiring induced defense mechanisms for protection against insects. Usually, the first steps upon herbivory recognition are the activation of kinase networks and biosynthesis of phytohormones (Maffei et al. 2012). As a consequence, defense mechanisms tend to interfere directly with insect growth and development, mainly via the activation of toxic proteins and metabolites, such as proteinase inhibitors and peroxidases (Govind et al. 2010). It has been proposed that most of the defense mechanisms against insect attack in plants are generally regulated by genes involved in the jasmonic acid (JA) and ethylene (ET) biosynthetic and signaling pathways (Wang and Wu 2013; Rehrig et al. 2014; Pangesti et al. 2016). JA- and ET-induced responses appear to be responsible for a large portion of the differential regulation of defense genes and regulatory elements, which can prime plants against biotic and abiotic stresses, improving tolerance.

The analysis of sugarcane transcriptome is a powerful approach to identify differentially expressed genes related to hormone biosynthesis and insect defense-related genes and to unravel the molecular mechanisms of tolerance to herbivory. Among available methods, RNA sequencing (RNA-seq) is a tool that allows the analysis of transcriptional profiles of any species of interest that present contrasting characteristics by quantifying the expression of genes activated or inactivated under certain conditions (Li et al. 2012). Despite the importance of sugarcane borer and the improvements in breeding programs related to borer tolerance, little is known about the molecular mechanisms related to resistance triggered upon *Diatraea saccharalis* infestation in sugarcane.

The absence of a reference genome imposed a drawback for further advances in understanding the molecular physiology of sugarcane. For long, the genome of the diploid species *Sorghum bicolor* had been used for this purpose because of the overall sequence collinearity and similarity with the sugarcane genome. However, because of the genomic complexity of its polyploid genome, the transcriptome analysis of sugarcane cultivars had been limited by the presence of allelic variation and a variety of transcript isoforms, which are difficult to identify using diploid genome models. Only recently, a reference transcriptome for sugarcane, named SUGIT (Sugarcane Iso-Seq transcriptome database), became available (Hoang et al. 2017). SUGIT is the first reference for complete transcripts sequences (full-length) of sugarcane and provides an excellent coverage of its transcriptome, contributing significantly for gene expression studies. More recently, a reference sequence of sugarcane monoploid genome became available (Garsmeur et al. 2018). This reference is a single tiling path (STP) which encompasses a single copy of the sugarcane gene space and contains 25,316 annotated protein-coding genes. STP and SUGIT are, respectively, a well-suited genome and transcriptome references for sugarcane gene expression studies.

Here, we analyze the transcriptional profiles of Brazilian sugarcane cultivars RB867515 and SP80-3280 in response to *D. saccharalis* infestation. These cultivars were chosen based on their susceptibility to the borer attack. In preliminary tests, both cultivars presented different behaviors regarding culm injury and young larvae survival on leaves (Tomaz et al. 2017). In general terms, RB867515 has relatively low survival of early-stage larvae feeding on leaves and is known to be more resistant in field conditions. Conversely, SP80-3280 is more susceptible to field and has lower mortality of early-stage larvae feeding on leaves, allowing then more larvae to penetrate the stalks. Thus, this study focused on characterizing the transcriptional changes of sugarcane and on identifying differences related to defense mechanisms possibly involved in the sugarcane tolerance to *D. saccharalis*.

Results and discussion

RNA-seq quality assessment

In summary, the samples obtained in this study consisted of good quality RNAs (Online Resource 1. Table S1). A reduced version of SUGIT containing 38,240 canonical sequences representatives of the transcriptome (SUGIT.UniRef80) was selected as reference for read mapping (see "Materials and methods"). Even with the complexity reduction, the number of SUGIT.UniRef80's transcripts was superior to STP's genes (25,316 sequences), which could represent isoform variants of genes, non-coding regions or translated regions still not annotated in the sugarcane reference genome. Besides, STP was developed based on sorghum genome, which might not be sufficient to capture all sugarcane gene fractions.

Approximately 4% of low-quality reads were removed from all RNA-seq libraries. The number of raw, filtered, and mapped reads are listed in Table 1. In general, more reads were mapped to SUGIT.Ref80 reference (69.5% average) than to STP's (58.6% average), probably because of differences in genetic backgrounds and the number of sequences included in each reference database. Despite having an overall lower percentage of mapped reads to STP's genes, the pairwise Pearson's correlation coefficient values ranged between 0.98 and 0.99 among the mapping outputs of read counts generated by STAR software (Online Resource 2. Fig. S1). Likewise, pairwise correlation values between 0.97 and 0.99 were observed among the normalized read abundances generated by Kallisto software (Online Resource 2. Fig. S2). These correlation values indicate that biological replicates data have good reproducibility.

Differential expression analysis

For comparison purposes, we adopted the terminology "differentially expressed transcripts" (DETs) and "differentially

Table 1 Summary of sequencing, trimming, and mapping of RNA-seq data in sugarcane cultivars RB867515 and SP80-3280

Description	Control			Infested		
	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
RB867515						
Total raw reads[a]	32,179,594	45,547,120	39,423,487	40,256,223	46,600,812	35,461,099
AfterQC filter[b]	31,817,945	44,961,584	38,986,933	39,871,276	46,245,737	35,217,036
Trimmomatic filter[b]	30,608,944	44,041,424	38,073,265	38,562,348	45,075,080	33,928,406
Trimming %	95.12	96.69	96.58	95.79	96.73	95.68
SUGIT mapped reads[c]	22,273,953	32,429,100	27,746,216	27,735,668	32,605,649	23,948,700
SUGIT mapping %	69.22	71.20	70.8	68.90	69.97	67.54
STP mapped reads[d]	19,038,128	26,251,435	22,790,464	23,027,575	26,282,287	20,445,196
STP mapping %	62.20	59.61	59.86	59.72	58.31	60.26
SP80-3280						
Total raw reads[a]	41,806,059	41,688,084	35,308,871	41,755,181	36,111,508	40,123,108
AfterQC filter[b]	41,367,001	41,238,701	35,029,655	41,317,273	35,678,615	39,832,180
Trimmomatic filter[b]	40,344,164	40,172,061	33,904,678	40,285,528	34,894,543	38,556,759
Trimming %	96.50	96.36	96.02	96.48	96.63	96.10
SUGIT mapped reads[c]	28,932,001	29,347,004	24,879,637	29,048,250	25,305,804	27,526,909
SUGIT mapping %	69.21	70.40	70.46	69.57	70.08	68.61
STP mapped reads[d]	24,229,774	22,002,610	20,190,748	23,619,686	20,599,366	23,108,662
STP mapping %	60.06	54.77	59.55	58.63	59.03	59.93

[a] Total number of reads obtained after Illumina sequencing

[b] Total number of clean reads obtained after quality filter using AfterQC and Trimmomatic

[c] Number of clean reads mapped to the transcripts of SUGIT database

[d] Number of clean reads mapped to the genes of STP database

expressed genes" (DEGs) ($\log_2|\text{ratio}| > 0.81$; FDR corrected P value ≤ 0.01) for the expression data obtained from SUGIT.UniRef80 and STP references, respectively. Differential expression analysis highlighted a more abundant transcriptional response for RB867515 when compared to SP80-3280, regardless of the mapping reference adopted (Fig. 1a–d). This result shows that RB867515 cultivar is more transcriptionally activated after 12 h of infestation, which could imply in quicker responses upon herbivory by probably triggering more defense-related genes and mediating metabolic pathways to cope with *D. saccharalis* attack. The complete list of overlapping and unique DETs and DEGs with their respective annotation and $\log_2$ fold changes ($\log_2$ FCs) is shown in Online Resource 1. Tables S2 and S3.

A reciprocal alignment between DETs and the coding DNA sequences (CDS) of DEGs was conducted to verify sequence similarity among them. The reciprocal BLAST searches showed that 255 DEGs significantly aligned with 298 DETs (Fig. 1 (E)). Surprisingly, 447 DETs and 161 DEGs had no matches in the alignments among differentially expressed sequences. When the BLAST searches were conducted between the entire set of SUGIT.UniRef80's transcripts and STP's CDS, there were still 230 DETs and 81 DEGs that had no significant matches. The complete list of the BLAST searches results between DETs and DEGs is shown in Online Resource 1. Table S4. This means that different sequences are considered differentially expressed depending on the reference adopted. These alignments provide an auxiliary tool for designing confidently specific primers in genes of interest, which could be further used in gene expression analysis in sugarcane. In addition, this information reveals that sequencing and annotation gaps or different genic regions still exist between SUGIT and STP references and ensures the need of using more than one reference database to capture a broader genetic background in gene expression studies with sugarcane.

Based on common herbivory responses and stress-related defense mechanisms (Howe and Jander 2008; Pandey et al. 2017; War et al. 2018), up- and down-regulated DETs and DEGs shared between the resistant (RB867515) and susceptible (SP80-3280) cultivars were selected and compared as for the relative $\log_2$ FC (Table 2). The selected genes are involved in hemicellulose, lignin, jasmonic acid (JA), ethylene (ET), and terpenoid biosynthesis, as well as in kinase, transcription factor, peroxidase, and chitinase activity. These comparisons

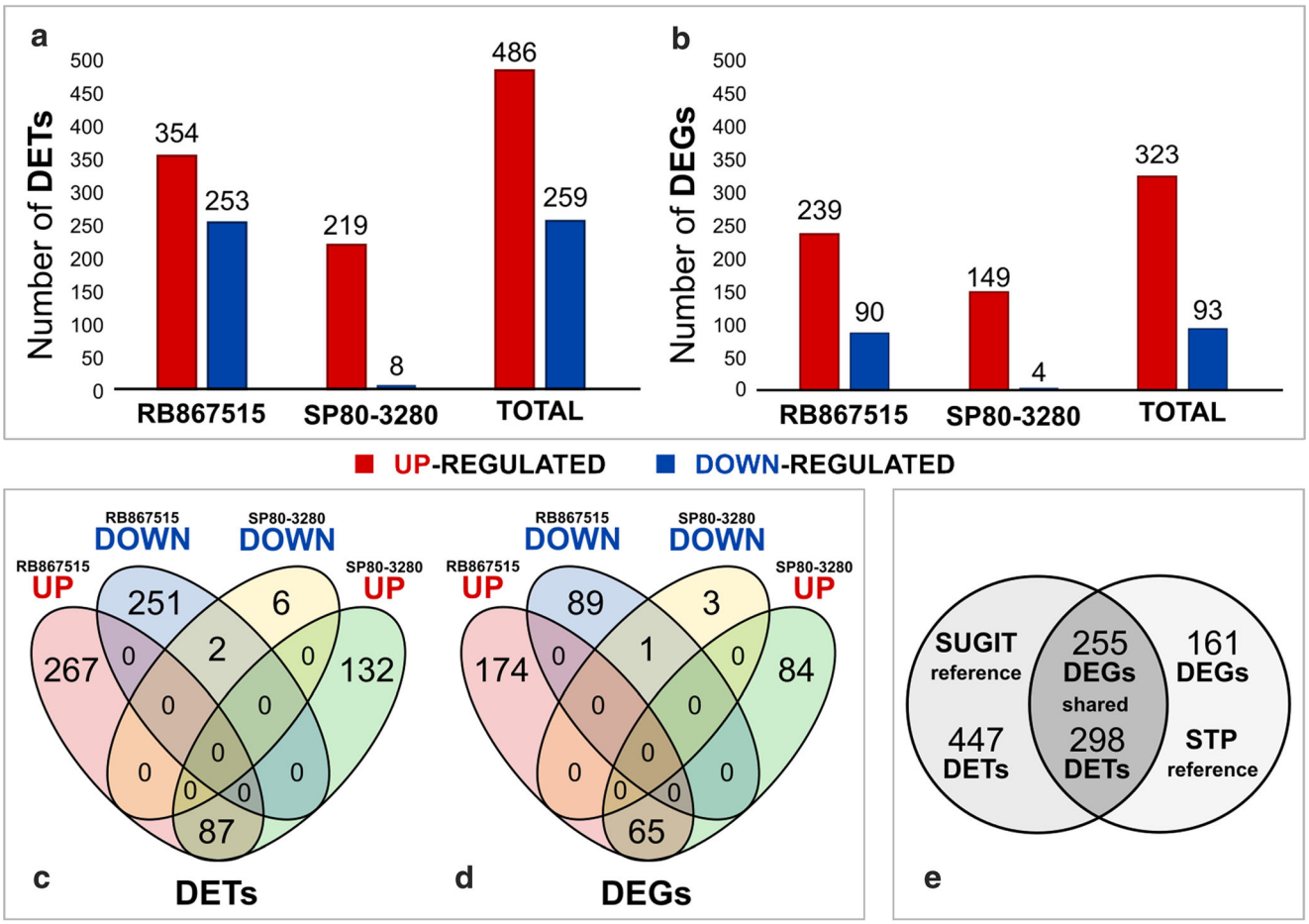

Fig. 1 Number of differentially expressed transcripts (DETs) and genes (DEGs) identified in sugarcane in response to *Diatraea saccharalis*. DETs and DEGs were identified after 12 h from infestation in sugarcane cultivars RB867515 (tolerant) and SP80-3280 (susceptible) using SUGIT.UniRef80 and STP databases as mapping references, respectively. (A and B) Number of up-regulated (red) and down-regulated (blue) DETs and DEGs found (FDR corrected *P* value ≤ 0.01). (C and D) Venn diagram of unique and common up- and down-regulated DETs and DEGs found in the cultivars RB867515 and SP80-3280. (E) Summary of reciprocal BLAST analysis results between DETs and DEGs

give insight into gene-specific regulation in each sugarcane cultivar in response to *D. saccharalis* infestation. Upon infestation, the expression of most of those genes increased significantly in both cultivars. However, genes involved in terpenoid and hemicellulose biosynthesis were up- and down-regulated, respectively, only in RB867515; whereas chitinase- and lignin-related genes were only up-regulated in SP80-3280.

In addition, a differential expression analysis among control samples of SP80-3280 and RB867515 was conducted to better understand their transcriptional background (SP vs. RB) (Online Resource 2. Table S17). Among the selected genes, only "cinnamoyl-CoA reductase" and "cellulose synthase-like protein E6" were differentially expressed, which means that their expression levels in the unstressed state are significantly higher in RB867515 (Table 2). Then, the difference in expression levels at the stressed conditionis attributed to how each cultivar mediates gene expression upon *D. saccharalis* infestation.

Functional analysis of DETs and DEGs

Sequence annotation

DETs and DEGs were functionally annotated through similarity searches to understand their possible functions in response mechanisms against *D. saccharalis* infestation. A total of 716 DETs (96.1%) and 403 DEGs (96.8%) were aligned to the non-redundant (nr) protein sequence database of NCBI using Blast2GO (Online Resource 2. Tables S2 and S3). Most of DETs and DEGs had significant alignments with genes from *Sorghum bicolor*, which is the closest-related diploid crop to sugarcane, and both share high sequence identity in their genic regions (Bundock et al. 2012).

In addition, TRAPID was used to assign conserved protein domains (PDs) to DETs and DEGs by comparing proteins encoded by their sequences with the InterPro database (Hunter et al. 2009) (Online Resource 2. Tables S5 and S6). TRAPID found 814 domains in proteins encoded by 723

Table 2 Log2 FC comparison of common and unique DETs/DEGs to RB867515 and SP80-3280. Similar regulation pattern observed in both mapping references for common DETs/DEGs in each cultivar

SUGIT ID	Log2 FC (RB867515)	Log2 FC (SP80-3280)	Log2 FC Controls (SP vs RB)	SUGIT annotation
GFHJ01047708.1	2.481235556	1.222585718	–	Putative linoleate 9S-lipoxygenase 3
GFHJ01024452.1	4.488776986	2.323548485	–	Putative WRKY transcription factor 71
GFHJ01053310.1	3.728606534	1.672803522	–	Mitogen-activated protein kinase kinase kinase 17
GFHJ01060610.1	5.106730053	3.877041972	–	Peroxidase N
GFHJ01058630.1	4.646091904	2.407987732	–	1-Aminocyclopropane-1-carboxylate oxidase
GFHJ01079377.1	5.694507527	–	–	Beta-sesquiphellandrene synthase-like
GFHJ01064943.1	–	1.506000369	–	Chitinase 2
GFHJ01067101.1	–	4.721071185	− 3.46746138	Cinnamoyl-CoA reductase 1
GFHJ01021685.1	− 1.143169041	–	− 0.87964499	Cellulose synthase-like protein E6
STP ID	**Log2 FC (RB867515)**	**Log2 FC (SP80-3280)**	**Log2 FC Controls (SP vs RB)**	**STP annotation**
Sh01_g010080	2.480729053	1.257895322	–	Linoleate 9S-lipoxygenase 1
Sh03_g015550	4.497950169	2.316476743	–	Similar to DNA-binding protein WRKY2-like
Sh09_g017390	4.838364907	1.886451567	–	Mitogen-activated protein kinase kinase kinase
Sh01_g036120	5.204327005	3.881502814	–	Peroxidase
Sh09_g004190	3.911033490	1.933901408	–	Acc oxidase
Sh07_g005540	5.681245474	–	–	(E)-beta-caryophyllene synthase
Sh09_g010120	–	1.497754937	–	Basic endochitinase A
Sh04_g026050	–	4.084322165	–	Cinnamoyl-CoA reductase-like protein 3
Sh02_g015820	− 1.203949150	–	− 1.31966354	Cellulose synthase-like protein E6

DETs (97%) and 592 domains in proteins encoded by 408 DEGs (97.8%) (Table 3). TRAPID also identified 421 and 282 different gene families among the annotated DETs and DEGs, having the "Plant peroxidase" and "Oxoglutarate family" as the most abundant gene families (GFs), respectively (Table 3). In general terms, peroxidases have been implicated in physiological processes that involve scavenging of reactive oxygen species (ROS) and synthesis of lignin and phytoalexins, which are important biological processes against biotic stresses (Cosio and Dunand 2009). Enzymes belonging to the oxoglutarate family participate in a variety of plant metabolic pathways, including the synthesis of hormones, signaling molecules and secondary metabolites (Cheng et al. 2014).

KEGG pathway and GO enrichment analysis

Several stress-related pathways and biological processes were enriched in both sugarcane cultivars and a similar pattern was observed in responses after 12 h of infestation with *D. saccharalis* (Online Resource 2. Tables S8, S9, and S11). Pathways such as "phenylpropanoid biosynthesis," "biosynthesis of amino acids,", "alpha-linoleic acid metabolism," and "linoleic acid metabolism" are known to be involved in plant stress responses and were significantly enriched and up-regulated in RB867515 and SP80-3280 (Fig. 2a and c). Pathways involved in "photosynthesis" and "carbon metabolism" were significantly enriched and down-regulated (Fig. 2b and d). Biological processes such as "response to wounding (GO:0009611)," "response to jasmonic acid (GO:0009753)," and "oxylipin metabolic process (GO:0031407)" are known to be associated to stress and plant defense were up-regulated in RB867515 and SP80-3280 (Fig. 3a and c). Only one biological process related to "photosynthesis; light harvesting in photosystem I (GO:0009768)" was observed as down-regulated in RB867515 (Fig. 3b). However, a greater number of down-regulated pathways and biological processes related to photosynthesis and carbon metabolism was observed in both sugarcane cultivars when genes showing small fold changes ($0 < \log_2|\text{ratio}| < 0.81$) were included in enrichment analysis (Online Resource 2. Figs. S3 and S4), suggesting that this transcriptional switch from an up-regulation of defense responses to a down-regulation of photosynthesis and carbon metabolism is still in a starting phase after 12 h of infestation.

Down-regulation of genes involved in photosynthesis has also been observed in rice under nematode (Wang et al. 2020) and bacterial pathogen mimic infection (Ranjan et al. 2015). In plants, photosynthesis and carbon metabolism might be compromised upon herbivory as a trade-off for the synthesis of defensive metabolites (Zhou et al. 2015). Down-regulation of photosynthesis-related genes is an adaptive response to biotic attack, which slow turnover of many photosynthetic

Table 3 Summary of TRAPID statistics for DETs and DEGs. Statistics displaying high gene similarity with *Saccharum spontaneum* and revealing oxoglutarate and peroxidase as the most representative gene families (GFs) among DEGs and DETs, respectively

Description	DEGs	DETs
Information	Number	Number
Total number	417	745
Average length (bp)	1039.8	1615.7
Similarity search information		
Saccharum spontaneum	293 (71.8%)	426 (58.9%)
Miscanthus sinensis	79 (19.4%)	193 (26.7%)
Sorghum bicolor	26 (6.4%)	56 (7.7%)
Zea mays	7 (1.7%)	21 (2.9%)
Setaria italica	2 (0.5%)	8 (1.1%)
Hordeum vulgare	–	5 (0.7%)
Zoysia japonica ssp. *nagirizaki*	–	5 (0.7%)
Cenchrus americanus	1 (0.2%)	3 (0.4%)
Lolium perenne	–	1 (0.1%)
Oryza sativa ssp. *indica*	–	1 (0.1%)
Oropetium thomaeum	–	1 (0.1%)
Phyllostachys edulis	–	1 (0.1%)
Triticum aestivum	–	1 (0.1%)
Triticum turgidum Svevo	–	1 (0.1%)
Total	408	723
Gene family (GF) information		
Gene families	282	421
Transcripts with GF	409 (98.1%)	723 (97%)
Largest GF	Oxoglutarate/iron-dependent oxygenase (10 genes)	Plant peroxidase (15 transcripts)
InterPro		
InterPro domains	592	814
Genes/transcripts with PD	408 (97.8%)	723 (97%)

proteins and allows plants to invest resources in immediate defense needs without debilitating near term losses in photosynthetic capacity (Bilgin et al. 2010). Furthermore, wound responses dependent of jasmonic acid also has been showed to promote the down-regulation of photosynthesis genes and a significant decrease in carbon assimilation (Havko et al. 2020).

As the pattern observed in differential expression analysis, RB867515 also showed a higher number of genes included in enriched pathways and biological processes than SP80-3280. RB867515 had 123 genes (considering DETs and DEGs) mapped to KEGG pathways and 186 mapped to biological processes GOs, while SP80-3280 had 70 and 68, respectively. When genes with small fold changes ($0 < \log_2|\text{ratio}| < 0.81$) are taken in consideration, the enrichment analysis also showed a different distribution of up- and down-regulated pathways and biological processes between RB867515 and SP80-3280 (Online Resource 2. Figs. S3 and S4). While SP80-3280 shows a predominant up-regulation of pathways and biological process, RB86715 shows a higher proportion of down-regulated genes involved in photosynthesis and sugar/carbon metabolism. It is also interesting to mention that the general GO term associated with "response to stress (GO:0006950)" showed opposite results for these genes with small fold changes. Meanwhile RB867515 starts showing a down-regulation of genes related to response to stress, SP80-3280 is still mediating the up-regulation of general stress-related responses against *D. saccharalis* after 12 h of infestation. Therefore, according to the enrichment analysis, we hypothesize that RB867515 responds faster than SP80-3280 and invests more resources in immediate defense mechanisms during *D. saccharalis* infestation.

Analysis of differentially expressed genes in control conditions (SP vs. RB) provide some insights that corroborate to this hypothesis. RB867515 showed an up-regulation of photosynthesis genes with enriched biological processes such as "photosynthesis, light harvesting (GO:0009765)" and "pyruvate metabolic process (GO:0006090)," while SP80-3280 showed an up-regulation of genes of oxidative metabolism with enriched biological processes such as "oxidation-

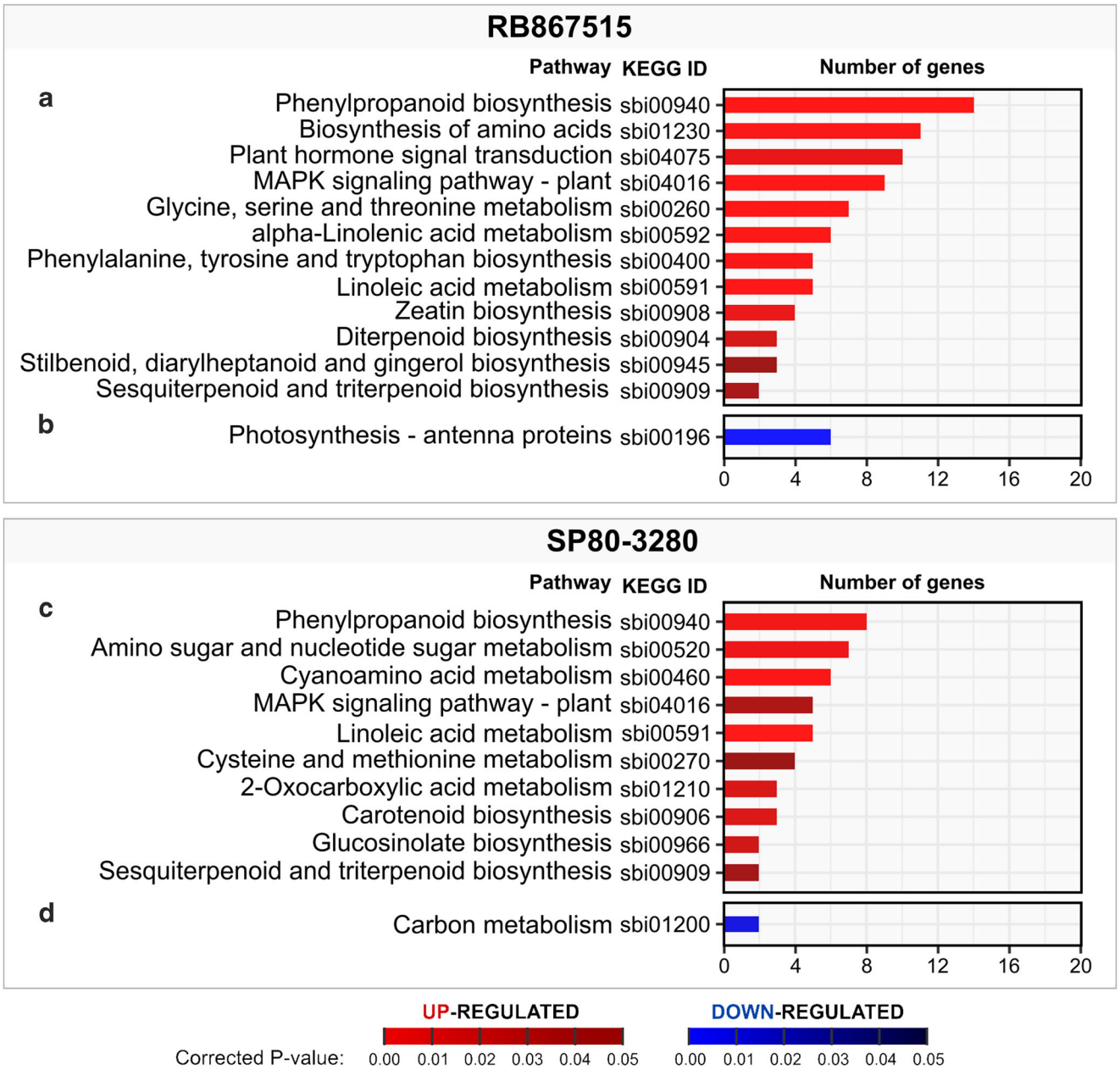

Fig. 2 Most enriched KEGG pathways identified in sugarcane cultivars in response to *D. saccharalis*. (A and B) Distribution of up-regulated (red) and down-regulated (blue) KEGG pathways assigned to cultivar RB867515 (tolerant). (C and D) Distribution of up-regulated (red) and down-regulated (blue) KEGG pathways assigned to cultivar SP80-3280 (susceptible). KEGG ID corresponds to reference metabolic pathways of *Sorghum bicolor*. The color scale represents the FDR corrected *P* value calculated in enrichment analysis

reduction process (GO:0055114)" and "glutathione metabolic process (GO:0006749)" (Online Resource 2. Table S16). These observations suggest higher transcriptional activity of photosynthesis genes for RB867515 at control conditions when compared to SP80-3280.

Another possibly related point in understanding the faster response and transcriptional activation of RB867515 upon herbivory when compared to SP80-3280 is the regulation of fructose-6-phosphate 2-kinase/fructose-2,6-bisphosphatase (F2KP) (Sh09_g005060; GFHJ01038535.1). F2KP was found as a DEG ($\log_2$ FC −0.65; FDR corrected *P* value $9.27e^{-6}$) and a DET ($\log_2$ FC −0.53; FDR corrected P value $5.01e^{-5}$) in comparison between controls (Online Resource 2. Table S17), which means that it is more expressed in RB867515 at control conditions. Fructose-2,6-bisphosphate is an important traffic signal in plant metabolism, and it coordinates the photosynthetic carbon flux into sucrose and starch metabolism, allowing plants to respond effectively to external conditions as a fast regulatory system of the ever-changing photosynthetic metabolism (Nielsen et al. 2004).

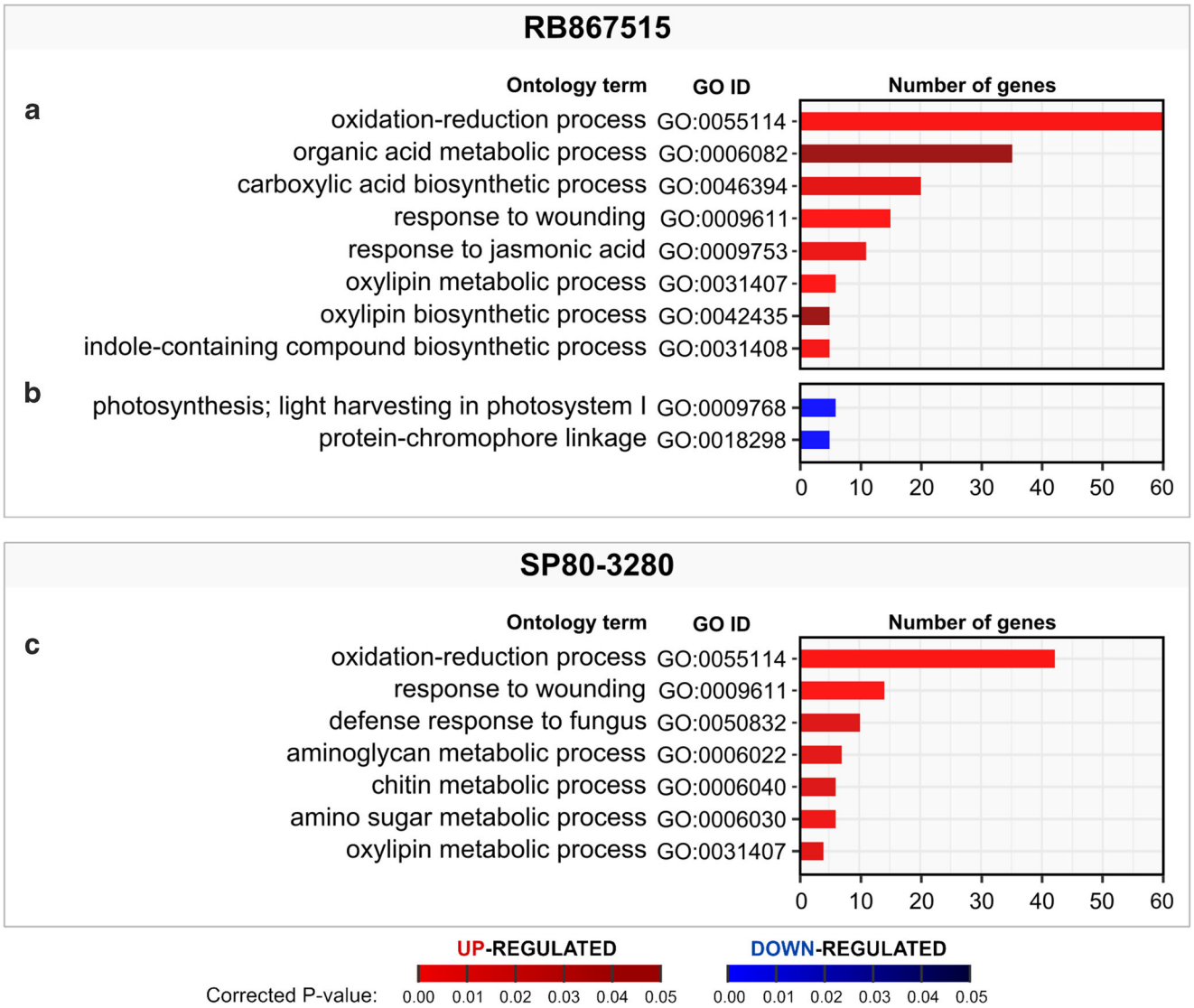

Fig. 3 Most enriched biological process Gene Ontology (GO) terms identified in sugarcane in response to *D. saccharalis*. (A and B) Distribution of up-regulated (red) and down-regulated (blue) GO terms assigned to cultivar RB867515 (tolerant). (C and D) Distribution of up-regulated (red) and down-regulated (blue) GO terms assigned to cultivar SP80-3280 (susceptible). The color scale represents the FDR corrected *P* value calculated in enrichment analysis

Regulation of genes involved in the biosynthetic and signaling pathways of ET and JA after *D. saccharalis* infestation

Phytohormones are usually implicated in defense mechanisms against pests. Basically, hormones participate in signal transduction pathways, promoting cellular crosstalk communication and coordination of transcriptome changes. Analysis of DETs and DEGs revealed that the signaling and biosynthetic pathways of ET and JA were upregulated in both cultivars, suggesting the involvement of these molecules in the defense responses during infestation with *D. saccharalis* in sugarcane herbivory (Online Resource 1. Table S12).

In infested treatments, key enzymes of the ET biosynthetic pathway, such as 1-aminocyclopropane-1-carboxylate synthase (ACS) and 1-aminocyclopropane-1-carboxylate oxidase (ACO), were upregulated (Fig. 4). These enzymes belong to the "cysteine and methionine metabolism." an enriched KEGG metabolic pathway upon *D. saccharalis* infestation in this study. ACS is an enzyme that catalysis the synthesis of 1-aminocyclopropane-1-carboxylic acid from S-adenosyl methionine (SAM), and ACO catalyzes the last step of the ET biosynthetic pathway by converting 1-aminocyclopropane-1-carboxylic acid to ethylene (Booker and DeLong 2015).

ET signaling genes were also up-regulated, and among the annotated DETs and DEGs are included: ethylene-responsive factor (ERF), reversion-to-ethylene sensitivity (RTE), ethylene response sensor 2 (ERS2).These genes were more predominantly triggered in RB867515 after *D. saccharalis*

infestation (Online Resource 1. Table S12). In general, ERS2 belongs to a family of membrane-associated receptors responsible for recognizing ET (Lacey and Binder 2014); RTE is involved in the regulation of ET receptors and is described as a negative regulator of ET responses (Resnick et al. 2008); and ERF transcription factors are involved in positively regulating ethylene and various stress responses (Xie et al. 2019). These genes are reviewed to be responsible for triggering defenses against abiotic and insect herbivory (Nguyen et al. 2016), but little is known about their interplay in sugarcane defense mechanisms against insect herbivory.

The JA and its derivatives have been recognized as key regulators in plant defense responses. The first steps in JA biosynthesis involve enzymes that participate in the "linoleic and alpha-linoleic acid metabolism," an enriched KEGG metabolic pathway in this study. During infestation with *D. saccharalis*, several DETs and DEGs belonging to the JA biosynthetic genes were identified, such as lipoxygenase (LOX), allene oxide synthase (AOS), allene oxide cyclase (AOC), and 12-oxophytodienoate reductase (12-OPR). These enzymes participate in the oxylipin biosynthetic process, and more precisely, they mediate the biosynthesis of JA and its derivatives from α-linoleic acid precursor via the octadecanoid pathway. The octadecanoid pathway has been reported to be involved in defense line mechanisms against biotic stresses (Santino et al. 2013). Here, LOX and AOS were upregulated in both cultivars, while AOC and12-OPR genes were up-regulated only in RB867515 (Fig. 4). Besides the similarities, according to the RNA-seq data, JA genes were more regulated in RB867515 in terms of expression level and number, an indication that the JA pathway is more activated in this cultivar upon herbivory (Online Resource 1. Table S12).

Genes involved in JA signaling transduction pathway, such as jasmonate ZIM domain-containing protein (TIFY/JAZ) and MYC2 transcription factor, were found to be up-regulated in this study. Belonging to the "basic-helix-loop-helix (bHLH)

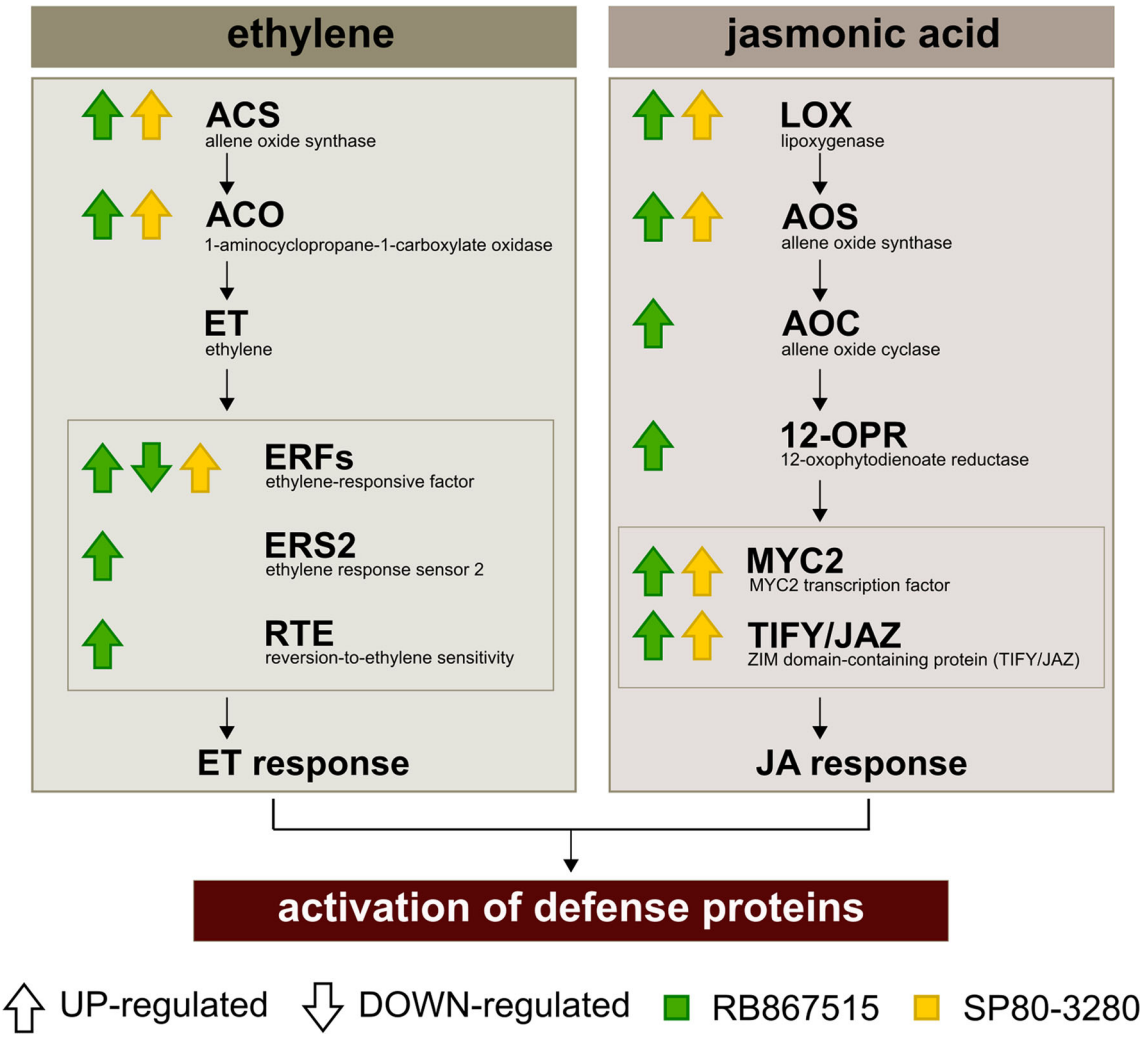

Fig. 4 General hormonal responses implicated in sugarcane infested by *D. saccharalis* infestation. Genes related to the biosynthetic pathways of ethylene (ET) (left) and jasmonic acid (JA) (right). Up- and down-pointed arrows correspond to up- and down-regulation, respectively. Green and yellow arrows correspond to regulation in sugarcane cultivars RB867515 and SP80-3280, respectively

related transcription factor" protein domain, MYC2 acts on the first line of signaling transduction after JA perception, positively modulating JA-dependent responses. Conversely, JAZ proteins are known to function as a repressor of MYC2 activity, posing a negative effect on the JA signaling pathway (Pauwels et al. 2010). Considering DETs and DEGs, more TIFY/JAZ genes were assigned to SP80-3280, whereas more MYC2 genes were assigned to RB867515 herbivory (Online Resource 1. Table S12). This indicates that RB867515 might have better control of the interplay between JA repressors and promoters.

This hormonal regulation pattern indicates that larval herbivory by *D. saccharalis* triggers similar JA and ET genes in the tolerant and susceptible cultivars used in this study (Fig. 4). However, some differences in number and regulation of DETs and DEGs assigned to these pathways were observed in both cultivars, which could represent different transcript isoforms of these genes and specific gene regulation that might be associated with herbivory-related defense mechanisms (Online Resource 1. Table S12).

Other reports highlight the transcriptional changes of JA and ET related to the plant's defense responses under caterpillar infestation. For instance, using transcriptome sequencing and analysis, similar hormonal regulation results were identified in sugarcane submitted to infection with the bacterium *Acidovorax avena*e subsp. *avenae* (Santa Brigida et al. 2016), in corn infested with the borer *Ostrinia furcanalis* (Yang et al. 2015; Wang et al. 2017), and in cotton submitted to *Hellicoverpa armigera* larvae infestation (Huang et al. 2015). Also, it has been demonstrated that JA possess a role in resistance against *Phytophthora cinnamomi* in maize (Allardyce et al. 2013) and against *Botrytis cinera* in strawberry (Jia et al. 2016).

It has been demonstrated that JA is involved in the synthesis of secondary metabolites including plant-defense proteins in certain species. For example, exogenous JA treatment induced the expression of pathogenesis-related genes (PRs) in rice (Yang et al. 2013). In sugarcane, JA-treated plants demonstrated certain resistance to *D. saccharalis* (Sanches et al. 2017), which could be associated with the regulation of defense proteins. Also, particular LOX isoforms can play important role in the activation of stress-induced defense responses in plants (Mariutto et al. 2011; Ogunola et al. 2017) and confer tolerance against wounding and insect attack (Wang et al. 2008). Thus, it is speculated whether different genes of LOX identified in this study, or possible isoforms, could regulate defense-related genes and impair different levels of tolerance in sugarcane.

These findings collaborate on the importance and involvement of JA and ET in the responsive mechanisms and regulation of signaling networks against biotic stresses. However, because of the polyploidy characteristic of sugarcane and the array of identified DETs and DEGs assigned to these pathways, further molecular investigation on single transcripts and genes could elucidate associations with specific phenotypic responses of sugarcane to *D. saccharalis* herbivory.

Regulation of defense-related proteins

Proteases and protease inhibitors

Generally, the recognition of insect elicitors by the plant's receptors triggers signaling transduction pathways that lead to the synthesis and accumulation of defense-associated proteins. Plant peptidase inhibitors are an example of plant-defense proteins produced upon herbivory. In this study, we identified a wide array of DETs and DEGs with a protease inhibitor annotation. In summary, annotations included "Bowman-Birk type bran trypsin inhibitor and Bowman-Birk type trypsin inhibitor (BBTI)," "Bowman-Birk type wound-induced proteinase inhibitor WIP1 (BBWIP1)," "Subtilisin Chymotrypisin inhibitor-2B (SCI-2B)," "Subtilisin Chymotrypisin inhibitor-2A (SCI-2A)," and "maize proteinase inhibitor (MPI)." Interesting, DETs and DEGs assigned to BBTI, BBWIP1, and SCI-2B were up-regulated in both cultivars; DEGs assigned to SCI-2A were only up-regulated in SP80-3280; and MPI were only up-regulated in RB867515, suggesting that these genes play a role in the defense against *D. saccharalis* attack.

Similar regulation patterns for these protease inhibitors have been reported for sugarcane under *D. saccharalis* artificial attack using the same hybrid cultivar SP80-3280 (Medeiros et al. 2016). In addition to the regulation pattern, the authors verified that Bowman-Birk inhibitor genes have different levels of expression when submitted to *D. saccharalis* attack and to wounding, suggesting that these proteins have a specific-triggering line of defense, which might be involved in the recognition of insect-specific elicitors. Up-regulation and accumulation of protease inhibitors have also been reported in maize infested with the leaf-eater *Spodoptera frugiperda* (Ankala et al. 2013), maize infested with the stem-borer *Ostrinia furcanalis* (Yang et al. 2015), and also in sugarcane after *D. saccharalis* infestation (Medeiros et al. 2012).

Besides the inhibitors, we also identified differentially expressed genes with a protease annotation: "serine carboxypeptidase II-3," "serine carboxypeptidase like-2," "cysteine proteinase 2," and "metalloendoproteinase 2MMP (MP)." In this study, MPs were only up-regulated by RB867515; serine carboxypeptidase like-2 and cysteine proteinases were only down-regulated in RB867515; and serine carboxypeptidase II-3 were up-regulated by both cultivars. Regulation of proteases and protease inhibitors with respective $\log_2$ fold changes is shown in Online Resource 1. Table S13.

Chitinases

Most of the up-regulated chitinase-related DETs and DEGs was mediated by SP80-3280 (Online Resource 1. Table S13), and "chitin metabolic process (GO:0006030)" was an enriched and up-regulated biological process for this cultivar (Fig. 3 (C)). It has been demonstrated that chitinases have an antifungal activity in plants, playing important role in defense mechanisms, inclusive in sugarcane (Tariq et al. 2018). Despite mostly studied for its antifungal properties, chitinases are also a protective agent against insects, attacking on chitin molecules that compose the insect's skeleton. For long, it has been verified an augment of chitinase expression under biotic stress, including insect attack, and this pattern has caught the attention to be involved in defense mechanisms. The defensive effects of chitinase genes have been reported in some species, including resistance of tomato against Colorado potato beetle (Lawrence and Novak 2006), of transgenic corn, expressing a chitinase gene, against *Spodoptera littoralis* (Osman et al. 2015), and of transgenic tea (*Camellia sinensis* [L.] O. Kuntze), overexpressing a class I chitinase gene from potato, against blister blight (*Exobasidium vexans*) (Singh et al. 2015). However, the role of the sugarcane chitinase family genes remain unclear due to the highly heterozygous and aneuploidy chromosome genetic background of sugarcane (Su et al. 2015).

Peroxidases

Several up and down differentially expressed sequences had a peroxidase annotation. In total, there were 15 DETs and 7 DEGs (Online Resource 1. Table S13). Interestingly, only RB867515 showed a down-regulation of POD genes. This regulation pattern and its annotation diversity suggest that different peroxidase genes, and probably different isoforms, are mediated for defense mechanisms in sugarcane upon herbivory.

It has been demonstrated that a peroxisomal catalase, another ROS scavenger enzyme, which also belongs to a multi-gene family, is responsive to biotic stresses and is suggested to be involved in the protection of sugarcane against oxidant-related environment stimuli (Su et al. 2014). Also, it has been suggested that lipoxygenases modulate ROS burst by consuming polyunsaturated fatty acids after wounding (Roach et al. 2015; Prasad et al. 2017), a process that could be associated with an increase of POD synthesis to counter-balance the negative effects of ROSs. The large number of POD genes in sugarcane, together with the diversity of processes catalyzed by peroxidases, suggest possible functional specialization of each isoform, but assigning a precise role for each individual peroxidase gene has continued to be a major bottleneck (Cesarino et al. 2012). Since *D. saccharalis* infestation resulted in differential expression of PODs, a closer-detailed investigation could reveal new insights into the function and specificity of peroxidase-related DETs and DEGs found in this work.

Cell wall biosynthesis

Lignin, cellulose, and hemicellulose are plant cell wall components that might dictate plant resistance to insect injury. Lignin accumulation plays an important role in the process of plant resistance to insects and can be used as a barrier directly or through the associated hormone signal pathway to increase insect resistance of plants (Liu et al. 2018). Genes involved in the biosynthetic pathway of lignin were regulated in this study. Cinnamoyl-CoA reductase (CCR), 4-coumarate:CoA ligase (4CL), and hydroxycinnamoyltransferase (HCT) are enzymes that participate in monolignol biosynthesis and were differentially expressed after *D. saccharalis* infestation (Online Resource 1. Table S12).

Most of the DEGs and DETs annotated to CCR, HCT, and 4CL were up-regulated by SP80-3280, indicating that this cultivar transcriptionally tends to reinforce its structures upon *D. saccharalis* infestation. On the other side, this result implies that RB867515 did not invest energy on morphological barriers to prevent larvae feeding, which is advantageous because lignin biosynthesis is not an immediate line of defense.

The enzymes "cellulose synthase A catalytic subunit 6 (CESA6)" and "cellulose synthase-like protein E (CSLE6)," which are involved in cellulose and hemicellulose biosynthesis, respectively, were also found to be differentially expressed. It has been shown that reduced levels of cellulose synthesis can lead to lignin accumulation mediated in part by JA and ethylene, leading to defense responses in *Arabidopsis thaliana* (Cano-Delgado et al. 2003). Besides regulating few biosynthetic genes of lignin, RB867515 also showed a predominantly down-regulation of cellulose synthase E and up-regulation of cellulose synthase A, demonstrating its transcriptional versatility to mediate the biosynthesis of cell wall components upon biotic stress (Online Resource 1. Table S13).

Validation of RNA-seq by RT-qPCR

RT-qPCR analysis was performed to corroborate with RNA-seq data (Fig. 5). Three DEGs involved in JA biosynthesis (LOX), protease inhibitor (SCI), and ferredoxin energy transport (FeS), and three DETs involved in JA biosynthesis (AOS), protease inhibitor (MPI), and hemicellulose biosynthesis (CSLE) were selected for that purpose. The assayed genes exhibited the expected positive or negative fold changes in qPCR reactions (Fig. 5a and d). However, SCI had significant up-regulation in qPCR for RB867515 (Fig. 5a), and MPI and CSLE exhibited significant up-regulation for SP80-3280 in qPCR (Fig. 5d). These genes did not differentiate statistically in RNA-seq data for the considered samples (Fig. 5b and e). We attribute these differences partly to non-controlled variation and to false-positive discovery correction in RNA-seq data because the difference in read counts mapped to each

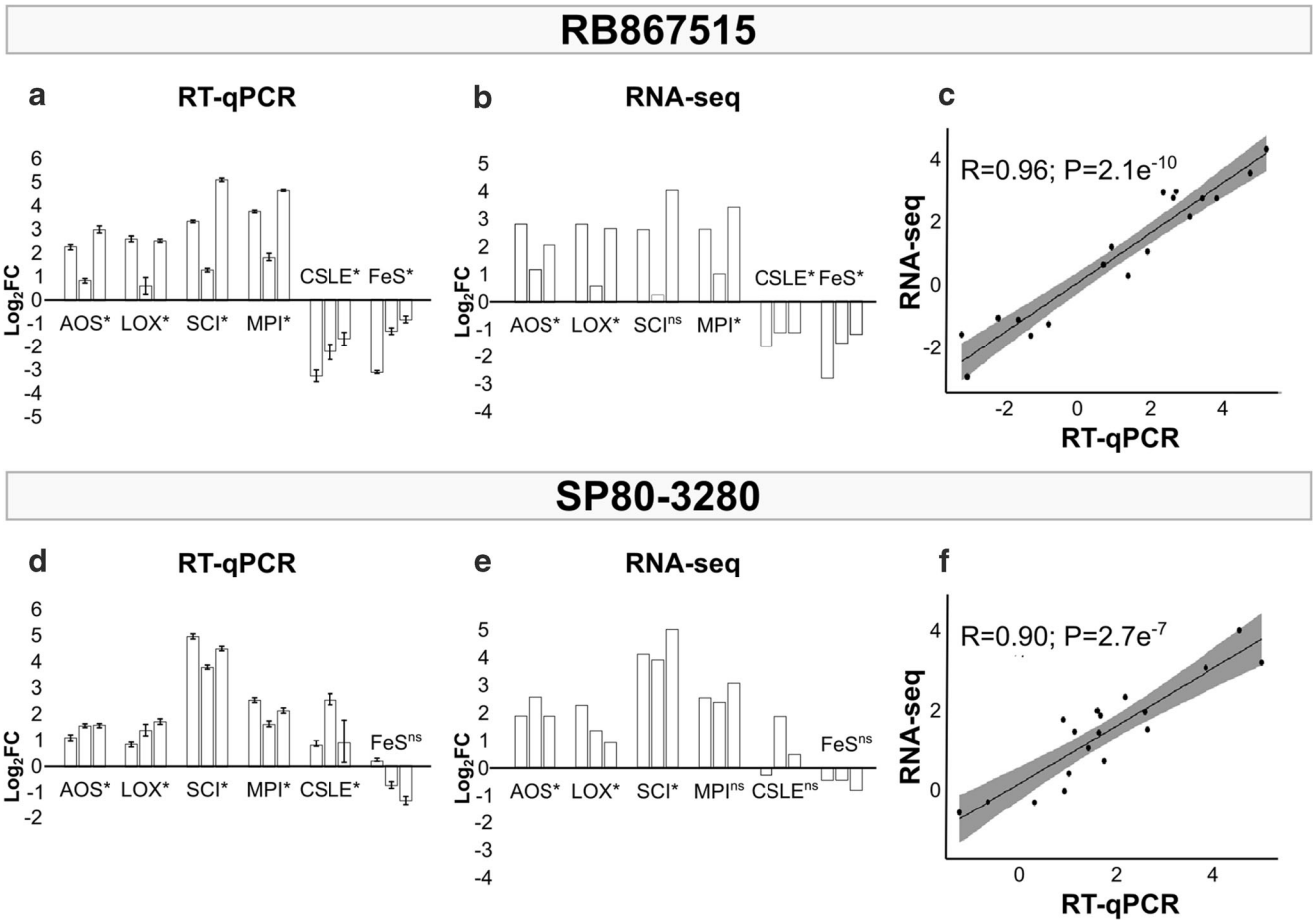

Fig. 5 RNA-seq analysis validation by RT-qPCR using candidate genes involved in different biological pathways. Three biological replicates were used. The bars of the RT-qPCR graphs represent the relative expression mean of three technical replicates (data = mean ± SD). (A and B) Relative expression levels obtained from RT-qPCR and RNA-seq analysis for RB867515 cultivar. (D and E) Relative expression levels obtained from RT-qPCR and RNA-seq analysis for SP80-3280 cultivar. (C and F) Pearson correlation coefficient analysis between log_2-transformed relative expression levels from RT-qPCR and RNA-seq results of RB867515 and SP80-3280 cultivars, respectively. *Statistical significance between control and infested treatments for RT-qPCR analysis (P value ≤ 0.05) and for RNA-seq analysis (FDR corrected P value ≤ 0.01); ^{ns}not significant for RT-qPCR analysis (P value ≤ 0.05) and for RNA-seq analysis (FDR corrected P value ≤ 0.01). LOX lipoxygenase, AOS allene oxide synthase, SCI subtilisin-chymotrypsin inhibitor, MPI maize proteinase inhibitor, CSLE hemicellulose synthase, FeS 3Fe-4S ferredoxin

gene in control and infested SP80-3280 samples leads to the regulation pattern observed in RT-qPCR, but did not represent statistical significance (FDR corrected P value ≤ 0.01) in RNA-seq. Despite the significance differences, RNA-seq and RT-qPCR data showed a high correlation for the selected genes, reaching R values of 0.96 and 0.90 for RB867515 and SP80–3280, respectively (Fig. 5c and f).

Conclusions

In summary, expression analysis highlighted a more abundant transcriptional response for tolerant cultivar RB867515 upon *D. saccharalis* herbivory when compared to susceptible cultivar SP80-3280, suggesting that it is more transcriptionally activated after 12 h from infestation, which may act as a tolerance mechanism to boost defense responses. Overall pathway analysis of both sugarcane cultivars showed an up-regulation of jasmonic acid (JA), ethylene (ET), and defense protein genes, as well as a down-regulation of pathways involved in photosynthesis and energy metabolism after *D. saccharalis* herbivory. However, RB867515 showed a higher proportion of down-regulated genes of metabolic pathways involved in photosynthesis and sugar/carbon when genes with small fold changes are considered, which suggests an immediate energy switch by probably allocating more resources to defense mechanisms in order to cope with the pest attack. In contrast to RB867515, SP80-3280 demonstrated a higher number of differentially expressed genes of metabolic pathways involved in lignin accumulation, probably to augment morphological structures upon infestation.

Materials and methods

Sugarcane cultivars, cultivation conditions, infestation, and experimental design

The sugarcane cultivars (RB867515 and SP80-3280) used in this work were obtained from RIDESA (Inter-University Network for the Development of Sugarcane Industry) (Barbosa et al. 2012). Culms from the cultivars were planted in 30 L pots filled with a mixture of 28 kg soil/manure (2:1 v/v) and grown in a greenhouse (average temperature 28.5 °C and natural light conditions) with soil humidity kept at soil water capacity. *D. saccharalis* larvae were obtained from a reserve colony grown in artificial diet (Hensley and Hammond 1968) with modifications (Araújo et al. 1985). The colony originated from a set of larvae collected in the field and grown in laboratory (Girón-Pérez et al. 2014). Sugarcane plants were evaluated using the leaf numbering system proposed by Kuijper (Kuijper 1915) and the leaves + 1 of individual plants were infested with 20 *D. saccharalis* larvae (3rd to 4th instar) after 2 months from the planting date, period in which plants were approximately 1 m high (Online Resource 2. Fig. S5).

The experiment was conducted in a randomized block design with two contrasting sugarcane cultivars (RB867515, resistant; and SP80-3280, susceptible), two treatments (control and infested), and five biological replicates. Three biological replicates of each experimental group were selected for RNA-seq analysis (Online Resource 2. Fig. S5A). The harvested vegetal material consisted of a section delimited by the bases of sheath and blade of + 1 leaf, including the internal leaves, which was collected after 12 h from the infestation. All samples were immediately frozen under liquid nitrogen after collection.

RNA extraction, RNA-seq library construction, sequencing, and bioinformatic analysis

Leaf samples were ground to fine powder under liquid nitrogen using stainless steel grind jars in the TissueLyser II (Qiagen) bead mill, and RNA was extracted using the PureLink® Plant RNA Reagent (Thermo Fisher) as described by the manufacturer. The quantification of total extracted RNA was accessed using a Qubit® 2.0 Fluorometer kit (Life technologies). The RNA integrity number (RIN) of the total RNA was analyzed using a 2100 Bioanalyzer 6000 Kit (Agilent Technologies, USA). A minimum of 10 μg of purified total RNA per sample with a RIN > 7.0 was sent out to GenOne Biotechnologies (Brazil) for constructing mRNA-seq libraries using paired-end mode (2 × 150 bp) in the Illumina HiSeq 2500 sequencer (Illumina).

The libraries underwent filtering steps to obtain clean reads, which included the following: (1) raw reads quality assessment using FASTQC version 0.11.8 (https://github.com/s-andrews/FastQC), and (2) trimming and removal of low-quality reads using AfterQC version 0.97 (Chen et al. 2017) and Trimmomatic version 0.38 (Bolger et al. 2014). After trimming and filtering, clean reads were mapped to the sugarcane monoploid genome database (STP) (Garsmeur et al. 2018) using STAR version 2.7.0a (Dobin et al. 2013) by selecting the quantification mode ("—quantMode") and default parameters. For complementary results, we also used the SUGIT transcriptome reference source (Hoang et al. 2017) to broaden the spectrum of read mapping. It is worth mentioning that the SUGIT database was constructed using a pool of RNA from different sugarcane genotypes under different stresses and from different tissues, which enhances the probability of catching broader transcriptional-triggered response profiles in sugarcane. Before mapping, the SUGIT database (107,597 transcripts) underwent a pre-processing step involving sequence complexity reduction to obtain a unified reference (UniRef) that was comparable to STP (25,316 genes) in differential expression analysis. For that purpose, CD-hit version 4.6.8 (Li and Godzik 2006) was used to cluster transcript sequences that share 80% of identity into canonical sequences representatives of the transcriptome. The UniRef80 was selected as that one with the number of canonical sequences closest to the number of genes of *Saccharum officinarum* (35,525 genes) and *Sorghum bicolor* (34,118 genes). Then, clean reads were mapped to SUGIT.UniRef80 using Kallisto version 0.45.0 (Bray et al. 2016) by selecting the quantification algorithm ("quant") with 1000 bootstrap samples ("–bootstrap-samples").

For terminology purposes, we referred to differentially expressed transcripts as DETs, and differentially expressed genes as DEGs according to the respective database adopted. The integrative analysis of DEGs and DETs concomitantly broadens the spectrum of the transcriptional profile of sugarcane and gives a better grasp of the biological processes triggered upon infestation, which enhances the exploitation of resistance traits in future studies and maximizes the understanding of how sugarcane responds to *D. saccharalis* infestation. A schematic overview of the analysis conducted in this study is shown in Online Resource 2. Fig. S6.

DETs and DEGs were identified using the DESeq2 package version 1.6.3 (Love et al. 2014) implemented in R version 3.5.1 (R Development CoreTeam (2018) 2018). DETs and DEGs analysis were conducted using the read mapping information generated by Kallisto and STAR, respectively. A false discovery rate (FDR) corrected P value ≤ 0.01 and a $\log_2$|-ratio| > 0.81 (|fold change| > 1.75) were used to select significant DEGs and DETs. In addition, Pearson's correlation coefficient analysis was performed to compare the read abundances from the mapping outputs obtained by STAR and Kallisto software. The read abundances were normalized as transcripts per million (TPM) and then transformed to $\log_2$.

This analysis included pairwise comparisons of the $\log_2$ transformation of normalized read abundances relative to all biological replicates in control and infested plants (see Online Resource 2. Figs. S1 and S2).

Characterization and functional annotation of DETs and DEGs

DETs and DEGs were functionally annotated using Blast2GO version 5.2.5 (Conesa and Götz 2008) through similarity searches using BLAST (McGinnis and Madden 2004) (Online Resource 1. Tables S2 and S3). The performed search parameters included: blastx-fast; nr database; taxonomy filter: monocots (taxa: 4447, Liliopsida); e-value: $1.0e^{-5}$; and number of Blast Hits: 5. Blast2GO was also used to perform a reciprocal BLAST analysis between DETs and DEGs using the following parameters: blastn (-task megablast); e-value, $1.0e^{-5}$; word size, 28; and HSP length cutoff, 75 (Table S4).

Conserved domains were assigned to the proteins encoded by DEGs and DETs using the online platform TRAPID: Rapid Analysis of Transcriptome Data (Van Bel et al. 2013) (Tables S5 and S6), which uses the PLAZA 4.5 database (https://bioinformatics.psb.ugent.be/plaza/) to assign functional annotations based on sequence similarity. The parameters of performed searches included the Poaceae database and an e-value of $1.0e^{-5}$.

Gene ontology (GO) terms were assigned to DETs and DEGs using the online platform PlantRegMap (Plant Transcriptional Regulatory Map) (Jin et al. 2017), which adopt the topGo package and Fisher's exact tests to find significantly overrepresented GO terms (P value ≤ 0.01). Sequences of DETs and DEGs were mapped to *Sorghum bicolor* genes using the ID Mapping tool of PlantRegMap (Table S7) and four lists of non-redundant Gene IDs were submitted to GO enrichment analysis: up- and down-regulated in RB867515, up- and down-regulated in SP80-3280 (Table S8). The lists of enriched Biological Process GO terms were further summarized using REViGO (Supek et al. 2011) (Table S9).

We also used KEGG Orthology Based Annotation System (KOBAS) version 3.0 to identify enriched metabolic pathways among DETs and DEGs (Xie et al. 2011). Sequences of DETs and DEGs were mapped to *S. bicolor* genes using the Annotation tool of KOBAS (Table S10) and four lists of Gene IDs were submitted to pathway enrichment analysis. Enriched KEGG pathways were determined by using Fisher's exact tests followed by Benjamini-Hochberg FDR correction method (FDR corrected P value ≤ 0.05) (Table S11).

Quantitative RT-PCR

The same three biological replicates for RNA-seq were used in RT-qPCR, but with independent RNA extraction. Total RNA was isolated using PureLink Plant RNA Reagent (Life Technologies). The extracted RNA was DNase-treated using TURBO DNase kit (Thermo Fisher) and, sequentially, the samples were submitted to heat (70 °C/15 min) as a final step for DNase deactivation. First-strand cDNA synthesis was performed from 500 ng of total RNA using RNA M-MLV Reverse transcriptase kit (Invitrogen). PCR reactions were performed in the QuantStudio3 instrument (Thermo Fisher) using the following program: 95 °C for 2 min, 40 cycles of 95 °C for 15 s, and 61 °C for 1 min, followed by melting curve. To each well was added a 10 μl final volume containing: 2 μl of diluted cDNA (1:25), 5 μl of GoTaq qPCR Master Mix (Promega), and 1.5 μl each primer (300 nM). Target gene expression was normalized using eEF-1a (eukaryotic elongation factor 1a) and eIF-4a (eukaryotic initiation factor 4a) as internal controls. Data were subjected to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). For statistical analysis, Student's t test (P value ≤ 0.05) was employed to compare the significance of the relative mRNA level of the selected genes between control and infested treatments. Pearson correlation test was employed to verify the correlation coefficient of $\log_2$ fold changes ($\log_2$FCs) between RT-qPCR and RNA-seq expression results. The design of primers was based on selected up- and down-regulation of DEGs and DETs in RNA-seq results with minor nucleotide correction according to read mapping. Primers are listed in Online Resource 1. Table S14.

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Author contributions USM and PMPV analyzed data and wrote the manuscript. PMPV, CEV, ACT, and MHPB planned and designed the research. USM, PMPV, CEV, ACT, and MF conducted the experiments. LAP and MHPB coordinated the research. All authors reviewed the manuscript.

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Data availability The RNA-Seq raw data are available at sequence read archive (SRA) (https://www.ncbi.nlm.nih.gov/sra), and this study was registered with the ID SRP243494 and BioProject ID PRJNA602327. The RNA-seq data of RB867515 (BioSample ID SAMN13892657) were registered with the following accession numbers: SRR10917298, SRR10917297, SRR10917294, SRR10917293, SRR10917292, and SRR10917291. The RNA-seq data of SP80-3280 (BioSample ID SAMN13892658) were registered with the following accession numbers: SRR10917290, SRR10917289, SRR10917288, SRR10917287, SRR10917296, and SRR10917295.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Code availability Not applicable.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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