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Sugarcane Serine Peptidase Inhibitors, Serine Peptidases, and Clp Protease System Subunits Associated with Sugarcane Borer (*Diatraea saccharalis*) Herbivory and Wounding

Ane H. Medeiros [1,2,†], Fabiana B. Mingossi [2,†], Renata O. Dias [2,†], Flávia P. Franco [2], Renato Vicentini [3], Marcia O. Mello [2,4], Daniel S. Moura [5] and Marcio C. Silva-Filho [2,*]

[1]   Departamento de Ciências da Natureza, Matemática e Educação, Universidade Federal de São Carlos, Araras, 13600-970 São Paulo, Brazil; anehm@cca.ufscar.br

[2]   Departamento de Genética, Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, Piracicaba, 13418-260 São Paulo, Brazil; fabiana.mingossi@gmail.com (F.B.M.); diasrdo@gmail.com (R.O.D.); flavia.esalq@gmail.com (F.P.F.); marcia.o.jose@gmail.com (M.O.M.)

[3]   Systems Biology Laboratory, Center for Molecular Biology and Genetic Engineering, State University of Campinas, Campinas, 13083-970 São Paulo, Brazil; shinapes@unicamp.br

[4]   Monsanto do Brasil, Campinas, 13069-380 São Paulo, Brazil

[5]   Departamento de Ciências Biológicas, Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, Piracicaba, 13400-918 São Paulo, Brazil; danielmoura@usp.br

*   Correspondence: mdcsilva@usp.br; Tel.: +55-19-3429-4442

†   These authors contributed equally to this work.

Abstract: Sugarcane's (*Saccharum* spp.) response to *Diatraea saccharalis* (F.) (Lepidoptera: (Crambidae) herbivory was investigated using a macroarray spotted with 248 sugarcane Expressed Sequence Tags (ESTs) encoding serine peptidase inhibitors, serine peptidases. and Clp protease system subunits. Our results showed that after nine hours of herbivory, 13 sugarcane genes were upregulated and nine were downregulated. Among the upregulated genes, nine were similar to serine peptidase inhibitors and four were similar to Bowman-Birk Inhibitors (BBIs). Phylogenetic analysis revealed that these sequences belong to a phylogenetic group of sugarcane BBIs that are potentially involved in plant defense against insect predation. The remaining four upregulated genes included serine peptidases and one homolog to the *Arabidopsis* AAA+ chaperone subunit ClpD, which is a member of the Clp protease system. Among the downregulated genes, five were homologous to serine peptidases and four were homologous to *Arabidopsis* Clp subunits (three homologous to Clp AAA+ chaperones and one to a ClpP-related ClpR subunit). Although the roles of serine peptidase inhibitors in plant defenses against herbivory have been extensively investigated, the roles of plant serine peptidases and the Clp protease system represent a new and underexplored field of study. The up- and downregulated *D. saccharalis* genes presented in this study may be candidate genes for the further investigation of the sugarcane response to herbivory.

Keywords: macroarray; sugarcane; *Diatraea saccharalis*; serine peptidase inhibitors; serine peptidase; Clp protease system; induced resistance; plant–insect interaction

1. Introduction

The sugarcane borer *Diatraea saccharalis* (F.) (Lepidoptera: Crambidae) is the most significant pest of sugarcane, with a wide distribution in Brazilian fields [1]. Sugarcane borer larvae create a vertical tunnel in the stem that becomes the primary route for microorganism entry [2]. The fungi that cause

stem rot, *Colletotrichum falcatum* (Went) and *Fusarium verticillioides*, are commonly found in the tunnels produced by *D. saccharalis*.

Plants recognize insect feeding via chemical interactions between self-molecules that may be modified by the insect [3] or via insect molecules [4,5]. This chemical interaction triggers a signaling cascade that causes the expression of defense-associated genes [6–9].

Plant peptidase inhibitors have been shown to inhibit digestive peptidases in herbivorous insects [10–12]. The induction and accumulation of peptidase inhibitors (PIs) after mechanical or insect wounding have been reported in several plant families and are considered part of the plant´s natural defense system against herbivory [10,13].

Bowman–Birk peptidase inhibitors were first isolated and characterized in soybean seeds [14,15] and were subsequently found in other leguminous plants [16,17] and in the Poaceae [18]. These proteins are associated with endogenous seed peptidases regulation, sulfur amino acids storage, and plant defense against pathogens and insects [19]. Their anti-nutritive function is derived from their formation of stable complexes with the catalytic sites of peptidases, blocking degradation and ingestion of amino acids from the insect diet [20,21].

Other than their involvement in responses to other types of stress, and in contrast to peptidase inhibitors, little is known about plant cytoplasmic and intra-plastid serine peptidases and their roles in defense against herbivory [22,23]. These peptidases have a housekeeping role in plants, releasing amino acids for recycling and eliminating non-functional proteins. However, peptidases have also been shown to play important roles in plant defense, acting in pathogen and pest recognition and in induction of effective defense responses [24].

Studies involving other plant species have elucidated the roles of various peptidases in plant defense; for example, in tomatoes, a serine carboxypeptidase was induced by wounding, systemin, and methyl jasmonate treatment [25]. Subtilisins have also been implicated in plant defense against herbivores [26,27]. There is evidence for a subtilase that is involved in systemin processing [28]. In tomatoes, systemin is synthesized in the form of a precursor, and following mechanical or herbivore injury, pro-systemin is processed and translocated throughout the plant, triggering a signaling cascade that ultimately activates defense genes such as proteinase inhibitors [29,30].

Non-serine peptidases have also been shown to be involved in resistance: Mir1-CP, a cysteine peptidase identified in maize *S. frugiperda*-resistant lines, is rapidly induced when plants areinjured [31,32]. The authors investigated why *S. frugiperda* larvae fed with maize leaves experienced diminished growth and found that this peptidase damages the peritrophic matrix of not only *S. frugiperda* but of various insects of the Lepidoptera order [33]. Leucine aminopeptidase A (LapA) is a late wound-response gene of tomato (*Solanum lycopersicum*) that accumulates after mechanical, insect and pathogen wounding [34–36]. Working in concert with LapA, arginase and threonine deaminase play roles in plant defense against herbivores as well [35,37,38].

Techniques such as macroarrays, subtractive libraries, AFLP-cDNA (Amplified Fragment Length Polymorphism-cDNA) display, and differential display RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) have enabled researchers to study changes in the transcriptome that are elicited in response to herbivory and wounding [39]. In contrast to research in other more widespread crops, progress in the field of sugarcane genomics has been slow. Genetic analyses are difficult in sugarcane due to its large and complex polyploid genome and the lack of sufficiently informative gene-tagged markers [40]. However, significant progress has been noted after the development of tools such as collections of expressed sequence tags (ESTs). Large EST collections have been made available [41–45] and have consequently renewed interest in sugarcane genetics [46]. Currently, the NCBI (National Center for Biotechnology Information) database lists more than 20,000 ESTs from the *Saccharum officinarum* complex (cultivated sugarcanes), and researchers have used these sequences to identify putative genes for the improvement of sugarcane field performance [47–49]. Using microarrays containing sugarcane ESTs, Rocha et al. [50] identified several sugarcane methyl jasmonate- and herbivore-responsive genes.

In silico analyses have shown that sugarcane possesses a set of conserved peptidase inhibitors that may also be involved in defense [19,48]. Initiatives to increase sugarcane borer resistance using traditional breeding and genetic engineering are needed. A better understanding of the sugarcane responses triggered by *D. saccharalis* feeding and wounding via the identification and characterization of genes directly involved in such responses may represent a means to improve sugarcane resistance. Those genes could also be used as molecular markers for insect resistance in traditional breeding programs.

In this study, we custom-made a macroarray containing 248 genes, including peptidase inhibitors, serine peptidases, and Clp protease system subunits, from the sugarcane EST collection. We have identified 10 peptidase inhibitors, seven peptidases, and five Clp subunits that are differentially expressed in sugarcane above-ground tissues in response to *D. saccharalis* feeding at an early time point.

2. Results

2.1. Macroarray Hybridization

To obtain information regarding the specific roles that peptidase inhibitors and peptidases may play in the sugarcane defense response against herbivores and wounding, a custom-made cDNA macroarray was constructed by spotting 248 selected ESTs on filter membranes (Table S1). The filter membranes were probed with ^{33}P cDNA populations derived from RNA extracted from the leaves of undisturbed sugarcane plants (0 and 9 h time points) and from the leaves of plants attacked by *D. saccharalis* (9 h time point). Probed membranes were effective for the identification of differentially expressed ESTs as exemplified by SacMPI-like1 (EST spots highlighted with a square in Supplementary Material Figure S1A–C) and SacBBI1 (EST spots highlighted with a circle in Supplementary Material Figure S1A–C). Both SacMPI-like1 and SacBBI1 exhibited stronger signals when probed with ^{33}P cDNA populations derived from the leaves of sugarcane plants attacked by *D. saccharalis* than when they were probed with populations derived from the leaves of undisturbed plants.

Of the 248 genes represented in the macroarrays, 22 presented consistent and reproducible expression in at least two hybridizations out of six possible comparisons (refer to Materials and Methods for more information regarding membrane randomization). Thirteen upregulated and nine downregulated genes were validated by quantitative real-time PCR; they are listed in Tables 1 and 2.

Table 1. Genes upregulated after 9 h of *D. saccharalis* feeding.

Sugarcane Clone Identification [a]	*E*-Value	Identity (%)	BLAST Hit [b]	Description [c]
SacBBI1 GI: 34966865 GB: CA113558.1	2×10^{-24}	73/87 (84%)	GI:195610004	Bowman–Birk type wound-induced proteinase inhibitor WIP1 precursor [*Zea mays*]
SacBBI2 GI: 35951517 GB: CA261007.1	1×10^{-40}	85/98 (87%)	GI:115434342	Bowman–Birk type proteinase inhibitor *Oryza sativa Japonica* Group
SacBBI3 GI: 35965021 GB: CA266304.1	5×10^{-43}	74/88 (84%)	GI:195610814	Bowman–Birk type bran trypsin inhibitor precursor [*Zea mays*]
SacBBI4 GI: 35984624 GB: CA272687.1	2×10^{-39}	85/98 (87%)	GI:115434342	Bowman–Birk type proteinase inhibitor *Oryza sativa Japonica* Group
SacMPI-like1 GI: 34922345 GB: CA070500.1	4×10^{-26}	51/60 (85%)	GI:214015177	Maize proteinase inhibitor [*Zea mays* subsp. *parviglumis*]
SacMPI-like2 GI: 35258606 GB: CA212876.1	6×10^{-33}	59/66 (89%)	GI:75994161	Maize protease inhibitor [*Zea mays* subsp. *parviglumis*]

Table 1. *Cont.*

Sugarcane Clone Identification [a]	*E*-Value	Identity (%)	BLAST Hit [b]	Description [c]
SacMPIlike3 GI: 36014330 GB:CA282462.1	1×10^{-31}	58/70 (83%)	GI:214015219	Maize proteinase inhibitor [*Zea mays* subsp. *parviglumis*]
SacMPI-like4 GI: 36037506 GB: CA288211.1	9×10^{-29}	55/65 (85%)	GI:214015093	Maize proteinase inhibitor [*Zea mays* subsp. *parviglumis*]
SacMPI-like5 GI: 36065043 GB:CA297188.1	2×10^{-28}	56/65 (85%)	GI:214015177	Maize proteinase inhibitor [*Zea mays* subsp. *parviglumis*]
SacCI-1B-like GI: 35010896 GB: CA129230.1	0.067	17/26 (65%)	GI:226507138	Subtilisin-chymotrypsin inhibitor CI-1B [*Zea mays*]
SacClp-like1 CA136349.1	2×10^{-77}	94/94 (100%)	GI:242061800	Clp amino terminal domain; *Sorghum bicolor*
SacChy-like GI: 35946811 GB: CA258670.1	1×10^{-153}	215/221 (97%)	GI:242077536	PDZ domain of trypsin-like serine proteases
SacCPD-like1 GI: 36002999 GB: CA278685.1				No significant similarity found

[a] GI: Gene Identification number and GB: GenBank Accession number; [b] BLAST hit was obtained using the BLASTX algorithm [51]; [c] Description indicates the putative functions of gene products expected from similar sequences.

Table 2. Genes downregulated after 9 h of *D. saccharalis* feeding.

Sugarcane Clone Identification [a]	*E*-Value	Identity (%)	BLAST Hit [b]	Description [c]
SacClp-like2 GI: 34940929 GB: CA087622.1	1×10^{-137}	192/198 (97%)	GI:195612324	ATP-dependent Clp protease proteolytic subunit 2 [*Zea mays*]
SacClp-like3 GI: 34966311 GB: CA113004.1	4×10^{-143}	208/224 (93%)	GI:413935895	Putative chaperone clp family protein [*Zea mays*]
SacClp-like4 GI: 35005555 GB: CA126553.1	5×10^{-62}	131/150 (87%)	GI:347602486	ATP-dependent Clp protease ATP-binding subunit ClpC homolog 1, *Oryza sativa Japonica* Group
SacClp-like5 GI: 35081269 GB: CA164148.1	7×10^{-108}	180/189 (95%)	GI:475585607	ATP-dependent Clp protease ATP-binding subunit clpA-like CD4A protein, chloroplastic [*Aegilops tauschii*]
SacCPD-like2 GI: 34966324 GB: CA113017.1	4×10^{-127}	177/198 (89%)	GI:195637388	Serine carboxypeptidase K10B2.2 precursor [*Zea mays*]
SacCPD-like3 GI: 35050806 GB: CA149102.1	7×10^{-58}	94/105 (90%)	GI:226507958	Serine carboxypeptidase K10B2.2 precursor [*Zea mays*]
SacSub-like1 GI: 34948297 GB: CA094990.1	2×10^{-136}	199/221 (90%)	GI:414880317	TPA: putative subtilase family protein [*Zea mays*]
SacSub-like2 GI: 34967468 GB: CA114161.1	2×10^{-42}	80/100 (80%)	GI:42407651	Putative subtilisin-like proteinase [*Oryza sativa Japonica* Group]
SacSub-like3 GI: 34967945 GB: CA114638.1	6×10^{-76}	124/158 (78%)	GI:475577050	Subtilisin-like protease [*Aegilops tauschii*]

[a] GI: Gene Identification number and GB: GenBank accession number; [b] BLAST hit was obtained using the BLASTX algorithm [51]; [c] Description indicated the putative functions of gene products expected from similar sequences.

2.2. Validation of D. saccharalis-Inducible Genes

To validate the expression of those genes upregulated after *D. saccharalis* feeding, we performed another set of biological experiments and included one more treatment: mechanical wounding. It was been shown that wounding, either by insects or mechanically, induces a general wounding response in plants [22,52–54]. In addition to that, insect wounding provokes a tailored response, specifically induced by some plant components released by insect feeding [3] or present in the insect saliva [53,55]. The objective was to compare the level of transcripts of 10 selected genes, after mechanical wounding and wounding by *D. saccharalis*.

2.2.1. Sugarcane Bowman-Birk Inhibitor (SacBBI) Genes

Four *D. saccharalis*-inducible sugarcane genes are homologous to rice and maize Bowman-Birk peptidase inhibitors and are designated here as SacBBI1-4 (Table 1). Real-time quantitative PCR analysis of the SacBBI genes showed that all four SacBBIs are induced by *D. saccharalis* and mechanical wounding (Figure 1). SacBBI1 is more responsive to mechanical wounding (17 times more highly expressed than the control) than caterpillar feeding (12 times more highly expressed than the control). SacBBI4 was induced by both caterpillar feeding and mechanical wounding; however, in contrast to SacBBI1, SacBBI4 mRNA levels in plants subjected to *D. saccharalis* feeding were approximately 600 times higher than the levels observed in control undisturbed plants and more than 50 times higher when plants were mechanically wounded (Figure 1). The other two genes, SacBBI2 and SacBBI3, were also induced by wounding and insect feeding, but to a lesser extent. Both the SacBBI2 and SacBBI3 genes were approximately 50 times more highly expressed in plants attacked by the insect and approximately 12 times more highly expressed in plants subjected to wounding.
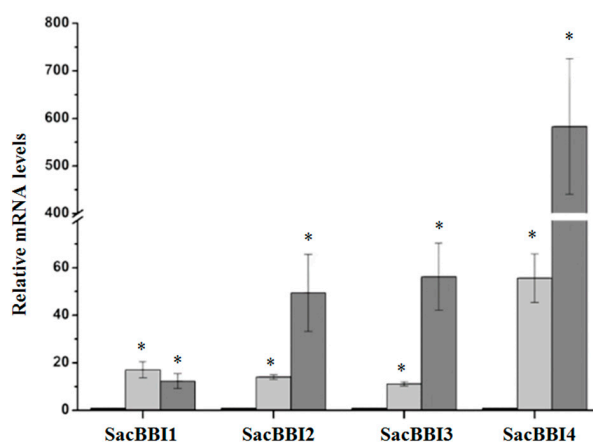

Figure 1. The relative expression levels of sugarcane Bowman-Birk peptidase inhibitors after 9 h of *D. saccharalis* feeding (dark gray bars), mechanical damage (light-gray bars), and control treatment (black bars). The expression levels were quantified by real-time quantitative PCR. The *x*-axis indicates the four sugarcane BBIs. The *y*-axis indicates the fold change in gene expression. The values are the means (±standard errors) of the transcripts from three replications, normalized to the transcript abundance of GAPDH. The regulation of expression was calculated using REST 2008 software [56]. The asterisks above the bars represent significant differences compared with the control at 0 h at a significance level of $\alpha < 0.05$.

We further investigated the behavior of all sugarcane BBIs present on the array, independent of their macroarray expression pattern. Gene expression quantification of 14 sugarcane BBI homologs confirmed that the four BBIs identified by our macroarray (SacBBI1 to 4) were the only BBIs induced by mechanical wounding or *D. saccharalis* feeding. The expression of the remaining 10 genes was unaltered, regardless of the treatment (data not shown).

2.2.2. Sugarcane Maize-peptidase-inhibitor-like (SacMPI-like) and Chymotrypsin Inhibitor 1B-like (SacCI1B-like) Genes

Changes in the mRNA levels of five sugarcane maize-peptidase-inhibitor-like genes (SacMPI-like1-5) and one chymotrypsin inhibitor 1B-like gene (SacCI1B-like) in response to *D. saccharalis* attack and mechanical wounding were evaluated. All SacMPI-like and SacCI1B-like genes were responsive to insect attack and wounding (Figure 2). Insect attack induced higher levels of SacMPI-like2 gene expression (28 times more highly expressed than in the control plants) than did wounding (seven times more highly expressed than in control plants). SacMPI-like 3 exhibited the highest gene induction by *D. saccharalis* feeding, with mRNA levels averaging 300 times greater than control levels when plants were damaged by *D. saccharalis* and approximately 60 times greater when plants were mechanically damaged. SacMPI-like1, 4, 5, and SacC1B-like were more responsive to mechanical wounding than insect attack.
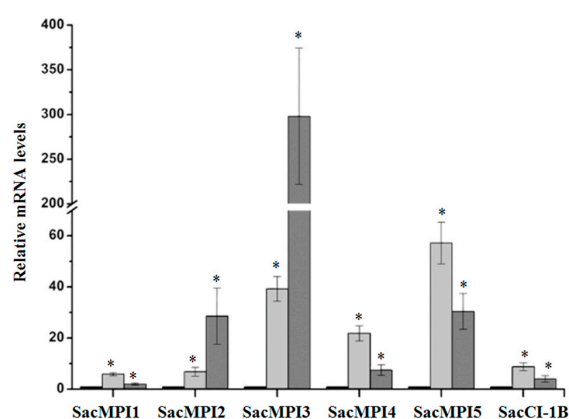

Figure 2. The relative expression levels of sugarcane maize-peptidase-inhibitor-like genes and the chymotrypsin inhibitor 1B-like gene after 9 h of *D. saccharalis* feeding (dark gray bars), mechanical damage (light-gray bars), and control treatment (black bars). The expression levels were quantified by real-time quantitative PCR. The *x*-axis indicates the six sugarcane serine peptidase inhibitors. The *y*-axis indicates the fold change in gene expression. The values are the means (±standard errors) of the transcripts from three replications, normalized to the transcript abundance of GAPDH. The regulation of expression was calculated using REST 2008 software [56]. The asterisks above the bars represent significant differences compared with the control at 0 (zero) h at a significance level of $\alpha < 0.05$.

All SacMPI-like genes are homologs of the I13 peptidase inhibitor family. To test the possible relationship between SacMPI-like gene evolution and its expression profile, we further investigated the distribution of all sugarcane SacMPI-like sequences within the I13 family via phylogenetic analysis (Supplementary Materials, Figure S2). I13 peptidase inhibitor homologs from sugarcane did not group based on their expression profiles (Supplementary Materials, Figure S2).

2.2.3. Serine Peptidases and Clp Protease System Subunits

Only two sugarcane serine peptidases and one Clp subunit were identified as induced by *D. saccharalis* in our macroarray, and their induction by herbivory was validated by performing quantitative real-time PCR analysis (Figure 3).

Among the 16 sugarcane Clp-like subunits analyzed in the macroarray, only the SacClp-like1 subunit (homolog of the *Arabidopsis* ClpD AAA+ chaperone subunit) was significantly induced by both wounding and insect attack ($p < 0.05$) (Table 3). Both SacChy-like and SacCPD-like1 were marginally induced by mechanical wounding. The insect wounding was not statistically different from control plants (at level of $p < 0.05$). SacClp-like1 gene was about 30 times more expressed in *D. saccharalis*-wounded leaves than in leaves of undisturbed control plants (Figure 3). Mechanical

damage caused an increase of the SacClp-like1 mRNA levels up to seven times the levels found in control plants.
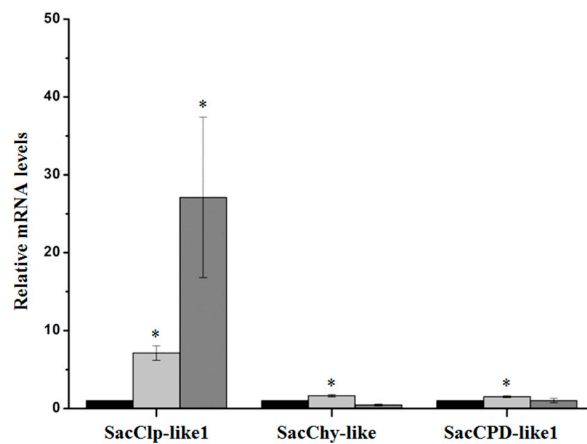

Figure 3. The relative expression levels of one sugarcane Clp subunit and two serine peptidases after 9 h of *D. saccharalis* feeding (dark gray bars), mechanical damage (light-gray bars), and control treatment (black bars). The expression levels were quantified by real-time quantitative PCR. The *x*-axis indicates the three sugarcane genes. The *y*-axis indicates the fold change in gene expression. The values are the means (±standard errors) of the transcripts from three replications, normalized to the transcript abundance of GAPDH. The regulation of expression was calculated using REST 2008 software [56]. The asterisks above the bars represent significant differences compared with the control at 0 (zero) h at $\alpha < 0.05$ significance level.

Table 3. Expression profiles of sugarcane Clp protease system subunit homologs after herbivore attack.

Role	*Arabidopsis* Subunit	Accession	Sugarcane EST Homologue	Expression Profile *
Clp AAA+ chaperones	ClpC1	AT5G50920	CA119085.1	-
			CA124181.1	-
			CA126553.1 (SacClp-like4)	-1.9 ± 0.07
			CA132637.1	-
			CA164148.1 (SacClp-like5)	-1.9 ± 0.06
	ClpC2	AT3G48870	CA113004.1 (SacClp-like3)	-6.4 ± 0.03
	ClpD	AT5G51070	CA119462.1	-
			CA136349.1 (SacClp-like1)	$27,11 \pm 10,31$
			CA145821.1	-
			CA194919.1	-
			CA212375.1	-
serine-type ClpP	ClpP1	ATCG00670	CA119497.1	-
	ClpP3	AT1G66670	CA119729.1	-
	ClpP4	AT5G45390	CA107353.1	-
	ClpP5	AT1G02560	CA074329.1	-
			CA183086.1	-
	ClpP6	AT1G11750	CA282400.1	-
	ClpP7	AT5G23140	-	-
ClpP-related ClpR	ClpR1	AT1G49970	CA108609.1	-
			CA108695.1	-
	ClpR2	AT1G12410	CA087622.1 (SacClp-like2)	-2.1 ± 0.14
	ClpR3	AT1G09130	Not found	
	ClpR4	AT4G17040	CA113615.1	-

* *p*-value < 0.005.

2.3. Validation of D. saccharalis-Repressed Genes

Nine sugarcane genes were repressed by *D. saccharalis* feeding, with the highest reduction observed for the homolog of the *Zea mays* serine carboxypeptidase K10B2.2 [57] (SacCPD-like3) (Table 4). The sugarcane homolog of the *Arabidopsis* ClpC2 chaperone subunit of the Clp protease system (SacClp-like3) exhibited a 6.4-fold reduction in mRNA levels in *D. saccharalis*-treated plants. A sugarcane homolog of a putative subtilase family protein of *Zea mays* [58] (SacSub-like1) demonstrated a 4.4-fold reduction in insect-attacked plants. The remaining six genes exhibited repression levels below 3-fold.

Table 4. Relative levels of gene repression after 9 h of *D. saccharalis* feeding.

Sugarcane Clone Name	GenBank Accession Number	Relative mRNA Level	*p* Value
SacClp-like2	CA087622.1	-2.1 ± 0.14	$p < 0.005$
SacClp-like3	CA113004.1	-6.4 ± 0.03	$p < 0.005$
SacClp-like4	CA126553.1	-1.9 ± 0.07	$p < 0.005$
SacClp-like5	CA164148.1	-1.9 ± 0.06	$p < 0.005$
SacCPD-like2	CA113017.1	-2.1 ± 0.07	$p < 0.005$
SacCPD-like3	CA149102.1	-44.2 ± 0.008	$p < 0.005$
SacSub-like1	CA094990.1	-4.4 ± 0.04	$p < 0.005$
SacSub-like2	CA114161.1	-2.7 ± 0.08	$p < 0.005$
SacSub-like3	CA114638.1	-1.6 ± 0.06	$p < 0.005$

3. Discussion

In this work, a customized macroarray containing 248 selected sugarcane genes putatively encoding serine peptidase inhibitors, serine peptidases, and Clp protease system subunits was used to identify sugarcane genes involved in the response to *D. saccharalis* herbivory. The macroarray technique used in this work has proven to be an elegant, rapid, and low-cost method to obtain the sugarcane transcript profile following *D. saccharalis* feeding. We identified 13 up- and 9 downregulated sugarcane homologs of serine peptidases and serine peptidase inhibitors (Tables 1 and 2).

Wounding of plant leaves, either by insects or mechanical injury, induces the rapid accumulation of peptidase inhibitors throughout the plant, in both damaged and adjacent tissues [10,49,59,60]. We hypothesized that when *D. saccharalis* feeds on sugarcane, it will trigger the accumulation of sugarcane serine peptidase inhibitors because *D. saccharalis* possesses an alkaline pH in its mesenterium, where serine peptidases are most active [20].

Our macroarray results showed that four sugarcane genes homologous to BBIs (SacBBI1 to 4) were induced in response to sugarcane borer herbivory (Figure 1 and Table 1). qPCR positively validated the expression of these genes and revealed that the increases in their expression ranged from 12 to 582 times the levels detected in undisturbed plants. The SacBBI2 gene was previously reported to be induced by insect and mechanical wounding [49]. The remaining 10 sugarcane BBI genes represented in the macroarray were not induced by *D. saccharalis* feeding. Studies examining the molecular evolution of this group have shown that the sugarcane BBIs can be divided into six subgroups based on amino acid sequence similarity [19]. Curiously, all four sugarcane BBIs induced by wounding diverge phylogenetically from the other ten BBIs that maintained constant expression. We speculate that our phylogenetic analysis, which grouped the BBI sequences into six groups, is accompanied by functional similarity. Some groups have diverged to fulfill a specific biological role in response to wounding (i.e., the four BBIs identified by our study), whereas other groups might possess other biological roles unrelated to plant defense (i.e., the 10 BBIs present in the macroarray that were not induced by wounding).

In addition to the four BBI genes that have already been discussed, our macroarray identified five genes with similarity to the potato inhibitor type I family, including the Maize Proteinase Inhibitor (MPI) and a gene similar to a subtilisin-chymotrypsin inhibitor (Figure 2 and Table 1). MPIs have been shown

to contribute to plant insect defense and are induced by insect and mechanical wounding [52,59–63]. The gene SacMPI-like2 CI-1 exhibits strong similarity to the subtilisin-chymotrypsin inhibitor CI-1B. In barley, the related chymotrypsin inhibitor family (CI-2) is associated with pathogen defense [64,65]. The inhibitors identified here may be candidates for incorporation into plant biotechnology programs. For example, when feeding on transgenic sugarcane overexpressing BBI and Kunitz-type PIs, *D. saccharalis* suffers diminished growth and metabolism [66]. In addition, *D. saccharalis* larvae raised on an artificial diet supplemented with peptidase inhibitors exhibit diminished growth and development and low fecundity rates [67,68].

Although serine peptidases and Clp subunits represented 81% of the genes present in our array, only two serine peptidases and only one Clp subunit were induced by *D. saccharalis* feeding (Figure 3 and Table 1).

Our results show that one putative sugarcane Clp protease system subunit (SacClp-like) was highly induced by herbivory. This subunit is homologous to the *Arabidopsis* Clp AAA+ chaperone subunit ClpD. Interestingly, three other sugarcane homologs of *Arabidopsis* Clp subunits were downregulated. The Clp protease system plays an important role in chloroplast protein homeostasis and metabolism [69], and *Arabidopsis* Clp subunits were previously reported to be involved in responses to light and cold acclimation [70,71].

The genes identified in this work can be further characterized and potentially used as molecular markers in sugarcane breeding programs [46] or as candidate genes for transgenic approaches for sugarcane improvement [40], which has been carried out for apples, tomatoes, and previously in sugarcane [66,72,73].

4. Materials and Methods

4.1. Plant Material and Insects

4.1.1. Phase 1—Sugarcane and *D. saccharalis* Experiments Used for Macroarray Hibridization

Sugarcane plants (*Saccharum* hybrid cultivar SP80-3280) were kindly provided by Centro de Tecnologia Canavieira (CTC), Piracicaba, SP, Brazil. Sugarcane plants were obtained from vegetative stalk cuttings called setts (nodal buds). One-eyed setts were planted in 200-mL plastic cups containing a commercial planting mix (Plantmax, Eucatex) and cultivated in a greenhouse at temperatures ranging from 18 (night) to 34 °C (day). *Diatraea saccharalis* was kindly provided by Centro de Tecnologia Canavieira, Piracicaba, SP, Brazil. The caterpillars were maintained on an artificial diet at 25 °C, 60% ± 10% relative humidity, and a 14-h photophase.

Biological experiments were conducted in the greenhouse facility of the Department of Genetics of the ESALQ (Escola Superior de Agricultura "Luiz de Queiroz"). To monitor sugarcane SP80-3280 transcript profiles after *D. saccharalis* attack, three treatments were applied: (A) non-attacked plants at the 0 h (time point); (B) non-attacked plants at the 9 h time point; and (C) *D. saccharalis*-attacked plants at the 9 h time point. Fourth-instar caterpillars were removed from diet and kept without food for 24 h before the beginning of the experiment. At this time, each caterpillar was carefully transferred with the aid of forceps, to the base of the stalk of each 20-day-old sugarcane plant. The feeding behavior of the caterpillar was checked for 20 minutes; if it failed to start feeding, the caterpillar was discarded and replaced. At the end of the experiment, the insects were removed. Each treatment had three biological replications, each consisting of a pool of four plants. After each time point, the entire aerial upper portion of the four plants of the replicate was collected, bulked, immediately frozen in liquid nitrogen, and RNA was extracted from this pool of plants. The entire biological experiment was repeated twice to confirm the first result.

4.1.2. Phase 2—qPCR Monitoring of Selected ESTs Identified through Macroarray

Expression of sugarcane ESTs identified through macroarray was validated through real-time qPCR. To perform this, another set of independent biological experiments was conducted.

The experiment was performed as described before (in phase 1), with few modifications. A mechanically-injured treatment was added. Thirty-day-old sugarcane plants were used, and the treatments were: (A) non-attacked plants at the 0 time point; (B) insect attacked plants at the 9 h time point; and (C) mechanically-injured plants at the 9 h time point. In the mechanical injury group, the plants were wounded repeatedly every hour with fine forceps, for 9 h.

4.2. Macroarray Construction

Our macroarray was built by the Brazilian Clone Collection Center using the sugarcane genes of 248 peptidase inhibitors, serine peptidases, and Clp protease system subunits selected from the SUCEST (Sugarcane EST Sequencing Project) database. The serine peptidases and Clp subunits comprised 81% of the total genes, whereas the peptidase inhibitors comprised 13% of the genes. The other 6% were represented by reference genes such as β-actin, GAPDH, eukaryotic initiation factor (eIF) and ubiquitin. The complete list of genes spotted onto the array is given in Supplemental Table S1.

The array was spotted onto six nitrocellulose membranes. The spot pattern consisted of a 3×3 array, and each sub-array contained two genes and an empty central spot. Each gene was spotted in quadruplicate. The macroarray technique used was established for filter-based methods [74].

4.3. Macroarray Normalization

To normalize the amount of DNA spotted on the filter membrane and to monitor the amount of DNA that was washed away after probe stripping as well as before and after cDNA probe hybridization, the macroarrays were hybridized with a probe designed to hybridize with a common region of the plasmid vector, specifically the Amp^r gene sequence [75] of the pSPORT1 vector, that is used to build the SUCEST libraries [41].

Probes were synthesized with the primers 5′-GTGGTCCTGCAACTTTATCCGC-3′ and 5′-TAGACTGGATGGAGGCGGATAA-3′ in the presence of [α-^{33}P] dCTP for 1 h at room temperature. After purification using ProbeQuant G-50 microcolumns according to the manufacturer's instructions (Amersham Biosciences, St. Catherine, ON, Canada), the probe was denatured for 3 min at 94 °C and added to the hybridization solution. All filters were placed in the same container, and pre-hybridization was performed for 4 h at 58 °C, followed by 18 h of hybridization (200 mL of 1% BSA, 0.5 mM EDTA (pH 8), 7% SDS, 1 M sodium phosphate (pH 7.2)). The filters were washed with decreasing concentrations of SSC. After washing, filter membranes were sealed with plastic film and were immediately exposed to imaging plates (Fuji Photo Film Co., Tóquio, Japan) for 72 h. Intensity signals were captured using a Storm 860 PhosphorImager (Bio-Rad, Berkeley, CA, USA). Next, the oligo vector probes were removed from the filters as described in [76]. The efficiency of probe removal was monitored by phosphorimager scanning after membrane filter exposition to imaging plates (Fuji Photo Film Co., Tóquio, Japan) for 72 h.

The median values of the signal intensities for all spots were determined. The variation coefficient of those values was estimated to assess the amount of DNA fluctuation among replicates [77]. Only those replicates with variation coefficients lower than 10% were used for subsequent analysis.

4.4. Probe Preparation

The filter membranes were probed with ^{33}P cDNA populations derived from RNA extracted from the leaves of undisturbed sugarcane plants (0 and 9 h time points) and plants attacked by *D. saccharalis* (9 h time point). To reduce variation among replicate filters, each of the three probes was hybridized, one at a time, with each of the three filter membranes over three rounds of hybridization. To do so, we

produced the three probes once, divided the preparations into three aliquots and kept them frozen until hybridization was carried out. After hybridization and the measurement of hybridization signals, only those genes that presented the same expression pattern (up- or downregulation) in at least two hybridizations among the six possible comparisons were selected.

To prepare the probes, total RNA from above-ground sugarcane tissue was used. RNA was extracted using TRIZOL® reagent (Invitrogen, Waltham, MA, USA), followed by deoxyribonucleic acid removal with two units of RNAse-free DNase I (Fermentas, Waltham, MA. USA) at 37 °C for 20 min. The RNA was re-extracted and then quantified by spectrophotometer. The RNA quality was checked by gel electrophoresis. Probes were produced by the reverse transcription of 30 μg of total RNA using SuperScript III (Invitrogen, Waltham, MA, USA) as well as 50 μCi of alphaP^{33}-dCTP and unlabeled dATP, dGTP, and dTTP, following the protocol of Schummer et al. [76]. The cDNA probes were purified by using ProbeQuant G-50 microcolumns according to the manufacturer's instructions (Amersham Biosciences, St. Catherine, ON, Canada). The probes were synthesized, purified, divided into three fractions, and frozen at −20 °C. For each cycle of hybridization, a new fraction was defrosted for use. Pre-hybridization, hybridization, and washing were performed as described in the Southern protocol [78]. After washing, filter membranes were sealed with plastic film and were immediately exposed to imaging plates (Fuji Photo Film Co., Tóquio, Japan) for 72 h. The intensity signals were captured using a Storm 860 PhosphorImager (Bio-Rad, Berkeley, CA, USA). Then, stripping was performed as described by Schummer et al. [76].

4.5. Macroarray Analysis

All signals were quantified using ArrayVision 8.0 rev 5.0 software (Imaging Research, London, ON, Canada). The grids were predefined and adjusted to obtain optimal spot recognition. For each spot, the AR volume (the spot density, minus artifacts and multiplied by its area), background (background pixel median density), and nARVOL (normalized AR volume) were measured. ArrayVision files were exported and opened using PMmA software [77]. Array normalization was performed with the Arrayflags script using the median of the overgo probe data. Only those genes for which the intensity signal average did not vary significantly among the four replicates were further used. Data analysis was performed using the algorithm ISER [79], which calculates and normalizes the signal intensity geometric average and ratio between the treatment and control. Genes were considered upregulated if the ratio was above the upper limit of the signal intensity threshold. Genes were considered downregulated if the ratio between the treatment and control signals was below the lower limit of the threshold.

4.6. Expression Analysis by Real-Time Quantitative PCR

Total RNA of each replicate was extracted with TRIZOL as described earlier in the macroarray section.

First strand synthesis was performed using Improm-II™ Reverse Transcriptase (Promega, Madison, WI, USA), according to the manufacturer recommendations, from 2 μg of total RNA, in a total volume of 20 μL. Real-time quantitative PCR was performed in a StepOne™ equipment (Applied Biosystems, Waltham, MA, USA) with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Waltham, MA, USA) reagent, following the recommendations of the manufacturer. The primer annealing temperature used was 62 °C and the fluorescence signal was captured at the end of each cycle. The melting curve analysis was performed from 72 to 99 °C, holding for 45 s during the first step and holding for 5 s during subsequent steps.

Primers for qPCR were designed based on the up- and downregulated genes. The Supplemental Table S2 presents the primer sequences used in this analysis. Data analysis was performed using the Pfaffl method [80]. The threshold was manually defined as 0.1 of the normalized fluorescence. Statistical analyses were performed using the Pair Wise Fixed Reallocation Randomization Test © [80] using 2000 randomizations and adopting $p < 0.05$ as the significance value.

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