Expression Profiles and Functional Analysis of Sugarwin1 in Sugarcane Genotypes in Response to Biotic Stress

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Abstract

Sugarcane is a worldwide acclaimed important agricultural crop utilized as a primary source of sugar and renewable energy in the form of biofuel. However, sugarcane plant production, vigor and yield, is usually constrained with constant biotic stress challenge such as pathogen infestation. Plant defense responses against pathogenic microorganisms are well catalogued in multiple plant species. Nevertheless, molecular details underlying such responses in sugarcane remain scarce, partly because of the genome complexity, which is highly polyploid. Thus, this study used qRT-PCR to evaluate sugarwin1 expression patterns among 10 different sugarcane genotypes originating from USA, Brazil and Pakistan. Among the 10 genotypes, sugarwin1 was highly expressed in US-658, US-633, and HSF-240; SPF-234, US-274, US-127, and US-641 had intermediate levels of expression; CPF-174, CPF-234, and CPF-77400 exhibited least or no expression level. The results presented herein demonstrating contrasting gene profiles suggest that sugarwin1 may constitute a common defense mechanism against biotic stress in sugarcane. These findings also imply that some genotypes of sugarcane might have evolved other intricate strategies to combat insect attacks and pathogen infections. More functional analyses of sugarwin1 gene profiles among different genotypes is critical to understanding how sugarcane tolerates stress and provides vital information to improve biotic stress resistance for sustainable crop production.

Keywords: Sugarcane; Sugarwin1; Biotic Stress; Expression Profiling; Genotypes

Introduction

Sugarcane (Saccharum officinarum L.) is an important sugar producing crops that is being widely used as a raw material in sugar production from many centuries Brazil on top. Sugarcane is classified under Saccharum L. genus, derived from intercrossing of S. officinarum (domesticated species with juicy, sweet, and thick culms), natural hybrids (S. barberi and S. sinense) and S. spontaneum (wild species). From several decades now, sugarcane stands as a fundamental alternative source in bioenergy production as the sugar-rich juice extracted from it in sugar industries produces ethanol which is consequently utilized in biofuel generation [1]. Sugarcane cultivation in world tropic regions produces approximately 1.3 billion metric tons of sugarcane stick production. Although sugarcane accounts for almost 30% of the world's sugar production, recent research findings have generated great interest in utility of sugarcane as a feedstock for biofuel production [2]. The increasing world's energy need, diminishing fossil fuel reserves, and the consequent upsurge in environmental pollution serves as a wake up call to the reality that human's bondage with fossil fuel cannot last much longer. In the light of these observed phenomena, it is crucial to intensify efforts on sustainable energy research which would lead to the invention of more environmentally friendly energy sources (biofuel) like sugarcane [3].

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Commercial sugarcane genotypes are poly-anneuploid hybrids having 100 - 120 chromosomes which vary from one individual cultivar to the other [4]. Genome size of sugarcane ranges from 926 to 760 Mbp which is comparable to sorghum (760 Mbp), and twice as large as that of rice (389 Mbp). Complexity of the genome has hampered potential use of molecular tools in developing sugarcane genotypes with improved tolerance and vigor to withstand biotic stressors [5]. Although traditional genetic breeding programs have been employed to achieve desirable traits, the usage of modern biotechnology techniques such as gene-targeted modifications to attain suitable agronomic phenotypes in sugarcane is still scarce [6]. The development improvement of modern genotypes will be achieved by characterizing genes relating to important agronomic traits such as biotic stress resistance [7].

Sugarcane yield is lagging behind due to constant attacks by innumerable biotic stresses which substantially reduce productivity [8]. Biotic stresses include fungal infections caused by Sporisorium scitamineum (sugarcane smut) [9]. Fusarium spp. and Colletotrichum falcatum (red rot) (Ashwin N, Barnabas L, et al. 2017); bacterial infections (red stripe) caused by Acidovorax avenae [10] and leaf scald (Xanthomonas albilineans) [11] sugarcane mosaic virus (SCMV) viral infection [12]. Insect pests, especially the sugarcane borer, Diatrea saccharalis, are the major culprits challenging sugarcane productivity by causing plant death resulting from apical death in young plants, lateral bud damage, weight loss, and frequent stem breakage in older plants. These attacks further expose these plants to opportunistic fungal, viral and bacterial infections which lowers both sugar and sugarcane alcohol production [8].

Plant responses to pathogens or herbivores attack involve complex mechanisms that modulate a multitude of defense-related genes. To effectively ward off invasion by pathogens and herbivorous insects, plants have evolved impressive mechanisms involving activation of defense related genes in leaves which neutralizes the digestive components of attackers. Novel in silico analysis studies enabled identification of sugarwin1 and sugarwin2, of barely which are homologues of barwin, widely characterized wound and pathogen-inducible protein [13] isolated from barley. Barwin domain is either found in association with other domains like chitin-binding domain as in the case of pathogenesis-related 4 (PR4) in tobacco and Arabidopsis, tomato P2, maize ZmPR4, rice OsPR4, and OsPR4b [14]. Wound-inducible genes (win) of numerous plant species have been described as protective against fungal infections such as F. verticillioides, Magnaporthe grisea and Fusarium culmorum [15]. Additionally, plants respond to environmental variations by eliciting an array of other cellular responses; phytohormones production is notably the most widely characterized plant defense system [16]. Cross-talk among hormone signaling pathways exhibiting either synergistic or antagonistic interactions significantly contribute to plant protection. Ethylene, jasmonic acid [17] and salicylic acid [18] constitute the key mediators of general and specific responses in plant immunity. In sugarcane, ethylene provides immunity against biotic stresses [19] during interaction with nitrogen-fixing endophytic bacteria [20].

Sugarwin1, a homologue of barwin in barley, is essential for wounding stress/biotic stress responses. Our findings show that few genotype showed higher expression of Sugarwin1 which provides a clue that these genotypes would be resistance to biotic stress and will have a bearing in breeding sugarcane genotypes that are better adapted to biotic stress across multiple environments. Wide array of high-throughput analyses has established plant gene profiles that mediate biotic stress responses. However, clear data defining crucial genetic markers for sugarcane improvement in response to biotic stress are very scanty. Thus, this study aimed to characterize and explore expression profiles of sugarwin1 in various sugarcane genotypes with a view to gain more insight into biotic stress responses.

Materials and Methods

Plant materials and cloning tools

Ten (10) different sugarcane genotypes plants were obtained from Sugarcane Research Station, Ayyub Agricultural Research Institute (AARI), Faisalabad, namely: US-274, US-641, US-127, SPF-234, CPF-234, HSF-240, US-633, CPF-174, CPF-77400, and US-658. Originally, US-274, US-641, US-127 and US-633 collected from USA, SPF-234, CPF-234, and CPF-174, CPF-77400 were collected from Brazil, and HSF-240 was an indigenous genotype in Pakistan. Stems collected from each genotype were used as the planting material. Expression vector pTZ57R/T and E. coli (K-12), which was used for the multiplication of sugarwin1 genes, were purchased from Thermo Fisher Scientific (USA). Restriction enzymes EcoRI and HindIII, T4 DNA ligase, Taq polymerase, PrimeScript RT-PCR Kit, in vitro cloning Kit, and RNase-free DNase were purchased from Invitrogen, Carlsbad (CA, USA). SYBR® Green PCR Master Mix Kit was purchased from Applied Biosystems (USA), and the CFX96 real time PCR detection system used to perform the reaction was purchased from BioRad (Hercules, CA, USA).

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**Cloning, sequencing and identification of sugarwin1 genes**

To obtain DNA sequences of sugarcane *sugarwin1* genes from the different genotypes, primer pairs were designed based on *win* gene sequences from barley and sugarcane EST (expressed sequence tag) resource from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Total RNA was extracted from sugarcane leaves using RNeasy mini kit (Qiagen, Valencia, CA, USA) following the manufacturer’s instructions [21]. cDNA template strand was synthesized from total RNA using the PrimeScript RT-PCR Kit (Invitrogen, Carlsband, CA, USA). Fragments of cDNA encoding open reading frame of the *sugarwin1* genes were amplified by PCR using specific primers (5’-GTCTGGTCAGTTAGGA-3’ and 5’-CTGGACTACGAGACGGTG-3’) designed using Primer Express 3.0 software. The PCR program was set at 95 ºC for 3 min for initial denaturation, 35 cycles for 1.5 minutes at 95ºC for final denaturation, 1.5 minutes at 57 ºC for annealing, 2 min at 72ºC for extension and the final extension was set at 72 ºC for 10 minutes. Genomic DNA was extracted from leaves following the cetyl-trimethyl ammonium bromide (CTAB) method as described by Allen., *et al* [22].

The amplified DNA sequence of *sugarwin1* was cloned into the pTZ57R/T cloning vector. Restriction analysis was carried out to confirm recombinant plasmid which was subsequently sequenced at (Molecular Biology Products Karachi, Pakistan). *Barwin* homolog (*sugarwin1*) was identified in the *win*-superfamily domain by NCBI blast (). Open reading frame (ORF) of the *sugarwin1* gene sequence was predicted using ORF Finder, an online tool obtained from NCBI (http://www.ncbi.nlm.nih.gov/orffinder/). nBLAST program was finally used for homology analysis.

**Phylogeny analysis**

Plant wound-inducible (*barwin*) protein sequences were retrieved from the GenBank database. The *barwin* protein sequences obtained were that of *Oryza sativa*, *Sorghum bicolor*, *Arabidopsis thaliana*, *Zea mays*, *Eucalyptus grandis*, *Jatropha curcus*, *Brachypodium distachyon*, *Setaria italica*, *Arachis duranensis* and *Morus notabilis*. A phylogenetic tree was constructed by alignment of retrieved protein sequences and that of *sugarwin1* obtained from the 10 sugarcane genotypes (Figure 2). Phylogenetic tree was designed using Neighbor-Joining method and bootstrap test based on MEGA 6.0 software.

**Expression analysis**

To analyze expression profiles of *sugarwin1* genes in sugarcane leaves, qRT-PCR (quantitative real-time PCR) was performed using RNA samples extracted from the different genotypes. The reaction was performed on CFX96 RT-PCR detection system (BioRad, Hercules, CA, USA). To amplify the *sugarwin1* gene, 5’-GTCTGGTCAGTTAGGA-3’ and 5’-CTGGACTACGAGACGGTG-3’ were used as the forward and reverse primers, respectively. The sugarcane GADPH (Glyceraldehyde-3-Phosphate Dehydrogenase,) gene (GenBank: CA254672) was used as a control to regulate the relative gene expression levels [23]. The qRT-PCR program was set at 95ºC for 30s for denaturation, 35 cycles for 10 s at 94ºC, 20s at 57ºC and 15s at 72ºC for amplification. Three replicates were set for every sample. The 2 \(^{-\Delta\Delta CT}\) method was adopted to calculate the relative expression levels.

**Statistical analyses**

Data presented in the current study denote mean ± standard deviation of three replicates. Statistical analyses were done by performing ANOVA test using SPSS software (19.0 version). Means were separated using post-hoc test, Duncan Multiple Range Test (DMRT), at 5% (P ≤ 0.05) significance level.

**Results**

**Cloning and structural analysis of sugarwin1 genes**

*Sugarwin1* clone was confirmed by performing restriction analysis using restriction enzymes (Online Resource 1). EST sequence of RT2 *Saccharum* hybrid cultivar SP80-3280 (accession number: CA145787.1) obtained from NCBI database showed 100% identity with the cloned nucleotide sequence of *sugarwin1* using nBLAST (Figure 1).
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Amino acid sequence and ORFs of *Sugarwin1*

*Sugarwin1* sequence analysis using the NCBI ORF Finder revealed 7 ORFs coding for 367 residues of amino acid and 6 stop codons (Figure 2). ORF4 was the longest ORF, coding for 107 amino acids while the shortest was ORF2 coding for 28 amino acids (Online Resource 2). ORFs 4, 5, 6 and 7 were found on the negative (-) strand while ORFs 1, 2 and 3 were located on the positive (+) strand. ORF4 (324 amino acids long) was used to predict protein structure (Figure 4).

![Figure 1: Homology of cloned sugarwin1 cDNA with the EST (expressed sequence tag) of RT2 Saccharum hybrid cultivar (SP80-3280) by using nBLAST [24].](image1)

![Figure 2: Nucleotide sequence and amino acid sequence of sugarwin1. *denotes stop codons [25].](image2)

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Phylogenetic analysis

Nineteen (19) different protein sequences were used to build the phylogenetic tree and analysis was performed to identify paralogues, xenologues and orthologues (Figure 3) [26]. Three different pairs of proteins were encoded by genes which are orthologues (win02_Sorghum bicolor and win02_Zea mays; win01_Zea mays and win01_Setaria italic; win01_Hordeum vulgare and win01_Brachypodium distachyon). This observation suggests that these genes share functional similarity and presumably evolved from the same ancestor. Similarly, two pairs of proteins, win02_Oryza sativa and win03_Oryza sativa; win01_Jatropha curcus and win01_Jatropha curcus, encoded by paralogues were identified, implying duplication and functional redundancy of genes. In addition, eight xenologues (win02_Setaria italic, win01_Sorghum bicolor, win01_Oryza sativa, win02_Zea mays, win04_Oryza sativa, win01_Eucalyptus gracilis, win01_Arachis duranensis and win01_Morus notabilis) were identified suggesting the occurrence of a speciation event. This could imply that genes coding for these proteins might have evolved due to horizontal gene transfer among different species. win01_Arabidopsis thaliana used as an out group was different from other groups of proteins probably because it lacks the identified Barwin conserved motifs (Figure 6).

Figure 3: Phylogenetic tree of Barwin protein in sugarcane and other plant species. The tree was generated using neighbor-joining method [27-29].

The accession numbers of proteins encoded by barwin in each plant species are: NP_001105464.2 (Oryza sativa); NP_001105462.2 (Zea mays); NP_001105461.2 (Sorghum bicolor); NP_001105460.2 (Brachypodium distachyon); NP_001105463.2 (Eucalyptus gracilis); NP_001105464.2 (Jatropha curcus); NP_001105465.2 (Arachis duranensis); NP_001105466.2 (Morus notabilis). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the tax analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acids substitutions per site. The analysis involved 19 protein sequences. All positions with less than 95% site coverage were eliminated. There were a total of 133 positions in the final data set. Evolutionary analysis was conducted using MEGA6 software.

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### Protein structure analysis

Phyre² (Protein Homology/analogy Recognition Engine) Version 2.0 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) was used to predict the protein structure using longest ORF (ORF4) of cloned sugarwin1. The predicted structure of the Sugarwin1 protein shown in figure 4 [30] has a length of 107 amino acids, 11.6 kDa molecular weight, and pI of 10 (Phyre²). N and C terminals were found at position 68 and 91 of amino acids sequence, respectively. *Sugarwin1* contains 6 common domains namely: Hydrolase domain, chitinases domain, Serine/threonine protein kinase domain, transcription factor IIA (TFIIA) beta-barrel domains, transferase domain, beta-beta-alpha zinc finger domain (Phyre²). Secondary structure of protein consisted of α-helix, β-strand, and a trans-membrane helix.

![Figure 4: Structural analysis of protein obtained from cloned sugarwin1 gene image colored by rainbow](image)

The model dimension is (+) X:19.746 Y:20.476 Z:11.827 [30,31].

### Expression profiles of sugarwin1

The analysis of sugarwin1 expression profiles through qRT-PCR indicated that the expression levels differed significantly across genotypes ($p < 0.0001$). Subsequent post-hoc for multiple comparisons using DMRT revealed that genotypes US-658, US-633, and HSF-240 highly expressed sugarwin1, genotypes SPF-234, US-274, US-127 and US-641 had intermediate levels, whereas CPF-174, CPF-234, and CPF-77400 exhibited the least expression profiles (Figure 5). Genotypes that had expression ranging from 2.5 to 3.3 were considered as highly expressed, those with 0.4 to 2.0 were classified as intermediates while those with < 0.4 expression levels were categorized as low/no expression levels.

![Figure 5: Expression profiles of sugarwin1 in leaves of different genotypes. Data denote mean ± SD of three replicates. G1 represents (US-274), G2 (US-641), G3 (US-127), G4 (SPF-234), G5 (CPF-174), G6 (US-633), G7 (HSF-240), G8 (CPF-77400), G9 (CPF-234), and G10 (US-658). Different letters indicate statistically significant differences in mean values at $p < 0.05$. Further differentiation was done by classifying 2.5 to 3.3 as highly expressed, 0.4 to 2.0 as intermediate expression and < 0.4 as low/no expression levels.](image)

### Citation

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**Conserved motifs analysis**

The structural diversity of *Sugarwin1* motifs was analyzed using MEME software (http://meme-suite.org/) (Figure 6). Different motifs are indicated by different color boxes with gray lines indicating non-conserved regions. Three motifs were common among all the proteins except for win01 *Hordeum* which has two conserved motifs, and win01 *Arabidopsis* with no conserved motif. The conserved motifs within these protein sequences suggest that *barwin* from the different plant species might have originated from same ancestral gene, and hence, the high homology.

![Figure 6: Motif distribution in Barwin. Different motifs are indicated by different color boxes while gray lines indicate non-conserved regions [32,33].](image)

**Sugarwin1 restriction mapping**

The restriction mapper online tool (http://www.restrictionmapper.org/) was used to explore the restriction map of cloned *sugarwin1* (Online Resource 6). The summary of the mapping is shown in table 1 below.

<table>
<thead>
<tr>
<th>Name</th>
<th>Conformation</th>
<th>Overhang</th>
<th>Minimum site length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugarwin1</td>
<td>Linear</td>
<td>5’ and 3’ blunt</td>
<td>5</td>
</tr>
</tbody>
</table>

*Table 1: Restriction map of sugarwin1.*

**Discussion**

Plants are frequently exposed to attack from a myriad of pathogens and herbivorous insects which mostly co-occur as a result of similarity of their terrestrial habitat [34]. To effectively combat these stressors, plants defense system has evolved to elicit responses that are commensurate with the intricate nature of invaders [35]. Plants react to pathogen attacks by inducing expression and accumulation of various pathogen-related proteins (Bravo JM, Campo S., *et al.* 2003) such as chitinases, peptidase inhibitors, glucanases, thaumatins, defensins, catalases, and glycoproteins [36]. The most widely characterized plant-induced defenses involve proteins encoding pathogenesis

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related (PR) genes [37] and they are regulated by multiple regulatory pathways [38]. Multiple studies have described barwin homologues in various plant species that play a crucial role in plant immunity against pathogenic infections. Several studies demonstrate that genotypes lacking barwin are more susceptible to microbial infections such as fungi attack [15,39].

The current study characterized sugarwin1 across 10 sugarcane genotypes with the view to extend the current understanding of the molecular mechanisms underpinning how various sugarcane genotypes build up robust immunity against fungal infections along insect herbivores [40]. Sugarwin1 and barwin are homologues that share antifungal properties [13]. The homology between sugarwin1 and barwin suggests that these genes encode similar protein structure with potential to perform related functions in various monocots and dicots [41,42]. A similar role in plant defense conferred by these proteins against pathogenic microorganisms has been reported [42]. Sugarwin1 has N-terminal beta-barrel domain and C-terminal chitinases binding domain sited after the signal peptide [30]. Analogous to Sugarwin1, Barwin proteins with conserved regulatory features have been implicated in plant defense mechanisms [43]. Previous research findings have implicated chitinases in plant immune responses to pathogens such as fungi, bacteria, and viruses [44]. Thus, the presence of chitin binding domain in Sugarwin1 protein further supports the hypothesis that it plays an important role in plant immunity [44]. Proteins under unfavorable conditions use various strategies to sustain stability. Research studies have cited that N and C terminal elements play a crucial role in modeling stability of proteins such as Barwin [45]. The close association reported between N and C terminal domains of Sugarwin1 and Barwin proteins therefore, implies that they play a primary role in stabilizing protein structure in extreme environments.

Wound-induced (WIN) genes are widely characterized in numerous plant species and constitute an essential component of plants’ defense system that is triggered upon perception of biotic stimuli. Contrasting gene expression levels reported among the genotypes suggest that among the highly expressed genotypes, sugarwin1 gene probably a constitutively expressed gene which activates early events of sugarcane defense responses. Alternatively, this finding points to a possibility that sugarwin1 may take part in general defense machinery against potentially harmful biotic stress factors such as fungi, bacteria, viruses, and nematodes. It could also be inferred that plant responses to unfavorable environmental cues vary by a range of mechanisms which control gene expression and enhances the rate of survival in such environments. Sugarwin1 anti-pathogenic activity specificity toward pathogenic fungi (for example, Ceratocystis paradoxa, Fusarium verticillioides, and Colletotrichum falcatum) has been widely reported. Several researches have indicated that Sugarwins have no deleterious effect on non-pathogenic fungi including Saccharomyces cerevisiae, and Aspergillus nidulans [46].

Collectively, these studies support the specificity of Sugarwin in reaction to pathogenic microorganisms in sugarcane plants. However, further studies assessing gene profiles among the genotypes in the context of fungal challenge would help to unravel the mechanisms behind the long hypothesized specificity phenomenon. Taken together, these research findings suggest that sugarwin1 may constitute a crucial component of fungi/insect-specific defense system; otherwise it serves a general immune function in other genotypes. Consequently, low expression of sugarwin1 in sugarcane is likely to down-regulate plant tolerance to insect herbivore attacks rendering them more susceptible to fungal infections. It is worth noting that sugarcane genotypes were collected from different regions, and thus our findings cannot exclude the possibility that environmental conditions might alter biotic gene profiles in sugarcane (Felix 2006). How environment may affect gene expression is however an open question. Wider sampling would be necessary to improve our understanding of sugarcane gene profiles [47].

Conclusion

Sugarcane is ranked highly among other crops with great agronomical and economical relevance worldwide, owing to its principal use in sugar and biofuel production. Relevant data about functional role of involved in sugarcane defense-related proteins remains scarce in comparison to other plant species, partly due to genetic complexity. Contrasting gene expression profiles of sugarcane populations explored in the current point to an interesting possibility that sugarcane defense might be genotype specific, and partly influenced by distinct environmental conditions. Thus, sugarwin1 may be utilized as a useful candidate gene for breeding sugarcane genotypes with superior resistance to biotic stress. Further in-depth research would provide deeper understanding into crucial genetic traits useful in breeding programs of sugarcane.

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