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MOLECULAR UNDERSTANDING OF RED ROT RESISTANCE IN SUGARCANE THROUGH GENOMICS AND PROTEOMICS

R.Viswanathan*, A. Ramesh Sundar, P. Malathi, V. Ganesh Kumar, M. Sathyabhama, M. Muthumeena and P. R. Rahul.

Sugarcane Breeding Institute, ICAR, Coimbatore 641007, India
*E-mail: rasaviswanathan@yahoo.co.in

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Abstract

Red rot of sugarcane caused by *Colletotrichum falcatum* Went is one of the devastating diseases of sugarcane causing significant loss to sugarcane production in the country. Complex polyploidy and lack of information on inheritance to red rot in sugarcane make breeding for red rot resistance more difficult. Studies were initiated at this institute on identifying red rot resistance mechanism in sugarcane. Studies conducted during the previous decades indicated possible role of oxidative enzymes, pathogenesis-related (PR) proteins and 3-deoxyanthocyanidin phytoalexins in governing red rot resistance. Recent studies involving semi-quantitative RT-PCR post pathogen challenge from sugarcane cultivars varying in red rot resistance, revealed differential accumulation of transcripts of the flavanoid biosynthetic pathway like coumarate-4-hydroxylase, chalcone synthase, chalcone reductase, flavanoid 3`-5` hydroxylase and flavanoid glycosyl transferase and this transcript analysis, further confirmed the role of sugarcane phytoalexins in red rot resistance. Similarly, the role of PR- proteins like chitinase and β-1,3-glucanase was established at the transcript level. Detailed molecular analysis applying differential display (DD)-RT-PCR identified expression of more number of differentially expressed transcripts during the host-pathogen interaction. Full length sequences of many potential transcripts were identified and are being functionally annotated. Also to identify specific proteins involved in red rot resistance, proteomics approach has been attempted by optimizing sample preparation from stalk tissues, 2-D electrophoresis (2-DE), down stream processing of identified spots and bioinformatics. Several resistance-associated proteins spots were identified and they are being analyzed critically for their functional relevance.

INTRODUCTION

Sugarcane (*Saccharum* spp. hybrids) is an important commercial crop grown in the Indian sub-continent and other countries. Sugarcane meets ~60% of the global requirement of sweeteners. India is the second largest producer of sugarcane in the world and the crop is cultivated in almost all the states occupying an area of 4.2 million ha with an annual production of ~300 million tonnes of canes. Sugarcane production in various regions of the country is affected by different biotic and abiotic stresses. Among the biotic constraints, red rot - a fungal disease caused by *Colletotrichum falcatum* Went (Perfect state: *Glomerella tucumanensis* (Speg) Arx Muller) is the major constraint affecting cane production in most of the sugarcane growing areas in Indian sub-continent, Thailand, Australia, Brazil, Fiji, USA etc. The disease occurs in 77 countries representing all the sugarcane growing continents worldwide(18,42). At present, the disease is prevalent in most of the sugarcane growing states in the country at varying intensities. The disease was first recorded in Java during 1893 and in India during 1901. Series of disease epidemics in the sub-tropical India had devastated cane cultivation in the entire Gangetic plains and Punjab till 1960s. Later the disease spread to parts of tropical India and caused severe damage to the crop resulting in the elimination of many commercial varieties, through disease epidemics which occurred in different regions (34,42). Severe epiphytotics on cv CoC 671- the wonder cane of

tropical region, during 1980s and 1990s have caused extensive damage to the cane industry in Peninsular India. Likewise many important commercial varieties BO 3, BO 10, BO 11, BO 17, BO 54; Co 312; Co 419, Co 453, Co 658, Co 785, Co 997, Co 1148, Co 1158, Co 6304, Co 7805, CoC 671, CoC 92061, CoJ 64, CoS 8436 etc were lost due to the disease (42). The repeated red rot epidemics brought heavy damage to the growers and millers in India.

In the early decades of the last century, breeding programmes in India were aimed at identifying varieties for subtropical India, to replace poor yielding Indian sugarcane *Saccharum barberi*. The breeding and selection process gave emphasis to adaptability, yield and quality improvement and resistance to red rot utilizing *S. officinarum*, *S. barberi* and the wild species *S. spontaneum*. The result was the release of outstanding varieties from SBI, which were high yielding and tolerant to the major disease - red rot. But recurrent outbreaks of red rot in epidemic forms resulted in the replacement of varieties which succumbed to this disease. Changing fungal race flora is considered as another major factor responsible for the breakdown of host resistance resulting in shorter life span of new varieties (25). In the absence of clear-cut information on inheritance of red rot resistance, exact mechanism governing red rot resistance in sugarcane remains yet to be understood clearly. However, two kinds of resistance *viz*., the structural or mechanical that is static and the physiological, which is dynamic, forms part of the plant defence against the pathogen. Only limited information is available on this area of work in sugarcane but for the work done at Sugarcane Breeding Institute (SBI), Coimbatore. This chapter summarizes red rot resistance with more emphasis on the recent findings on physiological resistance.

BIOCHEMICAL RESISTANCE

Different biochemical parameters that impart resistance in a particular genotype have been reported. Red rot resistant varieties in response to *C. falcatum* infection develop gum deposits that block the spread of the advancing hyphae by filling up the intercellular spaces. This process takes place in advance of infection and seals off further spread of the pathogen in adjoining tissues (20). Gum formation may also take place in susceptible

varieties, but to a lesser extent and usually after the tissue has been invaded which was termed as 'hypersensitive gummy reaction'. Later studies have reported on the role of phenolic compounds in red rot resistance. Higher quantities of phenols have been reported in resistant and moderately resistant varieties (16,44). However, further studies revealed that there was no correlation between total phenolic content and degree of resistance to red rot (3,17). Level of total phenols increased in resistant varieties after infection and it maintained, while in susceptible varieties, the level of phenolic content dropped after an initial increase. Number of host enzymes like peroxidase (POX), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and tyrosine ammonia-lyase (TAL) were reported to be associated with resistance in many sugarcane varieties (5,13,14,21,22)

PHYTOALEXINS

The reddish compound released in cells and intercellular spaces near the invading hyphae in the infected cane stalks is referred to as red rot pigment (RRP). The RRPs formed in response to *C. falcatum* infection in sugarcane inhibited conidial germination *in vitro* and slowed down the mycelial growth (3,29). Fractionation of the RRPs showed presence of several compounds and some of them are phenolics. Red rot pigments (RRPs) have been detected between 24 and 48h in the infected susceptible host (3) and it was found that conidial germination *in vitro* takes place much before the pigment accumulation. Hence, the pigment may not be effective in stopping the initial germination. The RRPs from resistant cane tissues showed seven compounds, while in susceptible cultivar CoC 671 has shown only four of them in thin layer chromatography (29). The loss of three pigment fractions in the susceptible cultivar after pathogen infection indicated that the invading pathogen in the stalks might have suppressed these resistance-contributing fractions.

In the chromatographic analysis, qualitative differences were not found between the resistant and susceptible varieties, before or after infection (3). Studies conducted at Sugarcane Breeding Institute confirmed the differential patterns of pigment accumulation between resistant and susceptible genotypes in response to pathogen infection. It was found that in resistant variety pigment localized at the site of inoculation at a

concentration of 1200 µg/g tissue, with little spread of pigment into the surrounding tissue, whereas susceptible showed a diffuse pigmentation throughout the pith with a lower concentration of 600 µg/g tissue. Studies conducted at the SBI also very clearly indicated that pigment accumulation concentrated in higher quantities around the site of inoculation in resistant varieties, whereas in the susceptible tissues the concentration was too low and in a diffused pattern. These studies are the firm evidences to show that a resistant mechanism operating in sugarcane against *C. falcatum*. Luteolinidin as the major component in RRP was identified and it appeared 24 to 48 hours after infection in cane tissues. Other components identified were luteolin, chlorogenic acid and a glucoside of luteolinidin. Fractionation of the pigments by high performance liquid chromatography (HPLC) revealed the presence of 3-deoxyanthocyanidin compounds, which were identified as luteolinidin, apigeninidin and caffeic acid ester of 5-0-apigeninidin (27,28). In resistant interaction, these compounds present at much higher concentration, whereas in susceptible or compatible reaction they were completely absent or present in very low concentration. Among the three compounds, luteolinidin level was the highest and present in all the varieties tested. Apigeninidin was also present in all the varieties but in low concentration.

Further studies revealed that susceptible variety accumulated reasonable amount of these phytoalexins, when toxin was used for inoculation, but in response to pathogen inoculation the same variety failed to synthesize phytoalexins probably due to their degradation by the pathogen. However, here also resistant variety recorded multifold phytoalexins synthesis than in the susceptible variety (28). The resistant variety may resist pathogen mediated phytoalexin degradation, thereby arresting the pathogen growth in the canes after pathogen inoculation. This was confirmed by inoculation of varieties varying in red rot resistance. Here induction of phytoalexins was recorded in both varieties. These findings clearly demonstrated that the phytoalexins were accumulated only in incompatible host pathogen interactions and compatible interactions had no such phytoalexins or with trace quantities. These three compounds were identified as phytoalexins in sorghum and *C. sublineolum* interaction, a host-pathogen interaction close to sugarcane and *C. falcatum* (19).

Subsequent studies with a set of host differentials proved that there is accumulation of anthocyanin compounds in incompatible interactions and no such induction/accumulation in compatible interactions (30,41). It has been conclusively proved that 3-deoxyanthocyanidin compounds act as sugarcane phytoalexins (6) and probably govern resistance in sugarcane to red rot. HPLC analysis with phytoalexin standards revealed differential accumulation of luteolinidin and apigeninidin phytoalexins in the cv Co 93009 resistant to red rot as compared to traces of compounds in the susceptible cv CoC 671.

The classical incompatible interaction of pathotypes from Co 1148 and Co 7717 on the host cultivars is very much demonstrated earlier at SBI, Coimbatore (7). This was further elaborated by quantifying phytoalexins in such interactions. The variety Co 1148 accumulates 3-deoxyanthocyanidins in incompatible interaction with Cf7717, which results in resistant reaction. However, the same variety fails to synthesize phytoalexins in case of matching pathotype Cf1148 infection. Similar behaviour of Co 7717 to Cf1148 and Cf7717 pathotype interactions was proved. Information on phytoalexin induction in susceptible varieties by phytotoxin also amply demonstrates the role of phytoalexin in red rot resistance in sugarcane. Though previous studies have been unsuccessful in pinpointing biochemical basis of disease resistance, these studies at SBI have shown the possible role of phytoalexins in red rot resistance.

PATHOGENESIS RELATED (PR) PROTEINS

Accumulation of PR-proteins is an important phenomenon of plant defence responses upon infection by pathogens. Many of the PR-proteins have been purified and the purified proteins exhibited strong antifungal activity against many fungal pathogens under *in vitro* conditions. Induction of high levels of these proteins in host plants reduced disease development in many crop plants. Specific involvement of many proteins in disease resistance has resulted in isolation and cloning of these PR-proteins and subsequently, developing transgenic plants expressing PR1a, PR-2 (β-1,3-glucanase), PR3 (chitinases) and PR5 (thaumatin-like proteins) in various crop plants (23). Such transgenic plants expressing foreign genes showed enhanced resistance to fungal pathogens. Detailed studies on PR-proteins in red rot resistance showed that

constitutive activities of chitinase and β-1,3-glucanase were higher in disease resistant varieties as compared to susceptible varieties. Upon pathogen inoculation, the resistant variety accumulated higher hydrolytic enzymes as compared to susceptible cultivars (34). This information suggests a possible role of these enzymes in red rot resistance.

Further studies were conducted to identify the PR-proteins using Western blot technique in a set of resistant and susceptible varieties differing in resistance to the disease (26). The red rot resistant cv Co 93009 showed differential induction of four chitinase proteins with molecular mass ranging from 34-39 kDa after pathogen inoculation in leaf tissues. The intensity of these proteins increased with time from 6 to 42 h after inoculation (Fig. 3). In susceptible variety CoC 671, induction of a 35-kDa chitinase protein was recorded. In stalk tissues, induction of a 35-kDa chitinase protein was recorded 24 h after inoculation in resistant variety whereas, in susceptible plants such induction was delayed. Similarly, early induction of 43 and 37.5kDa TLPs by 24 h was observed in response to pathogen inoculation in the resistant variety, whereas in the susceptible variety such induction was less intense and could be seen 9 days post inoculation. These studies gave a clear indication that the PR-proteins may form part of the important defense mechanisms operating in sugarcane against red rot as in other host-pathogen interactions.

MECHANISM OF INDUCED SYSTEMIC RESISTANCE IN SUGARCANE

Earlier studies have clearly established the induction of systemic resistance in sugarcane against red rot by fluorescent pseudomonads (37,40). These studies gave evidence of enhanced resistance in disease susceptible cultivars like CoC 671, CoC 90063, CoC 92061 etc under controlled and field conditions. Studies were conducted in detail on the mechanism of induced resistance mediated by strains of *Pseudomonas* against the disease. Involvement of different PR-proteins such as β-1,3-glucanases, chitinases and thaumatin-like proteins (TLPs) was found to be associated with *Pseudomonas*-mediated induced resistance (31,35) Chitinases purified from systemically protected stalk tissues of sugarcane against *C. falcatum* showed strong anti-fungal activities (31). It has also been clearly demonstrated that

disease susceptible sugarcane plants following treatment with *Pseudomonas* sp. is able to restrict disease development to a level equivalent to moderately resistant varieties and many PR-proteins are involved in that ISR (Fig. 4). In addition, enzymes of phenyl-propanoid pathway and oxidative pathway were also found to be involved in ISR (38). Characterization of *Pseudomonas* strains revealed that production of different metabolites/antibiotics such as salicylic acid, auxins, siderophores, pyocyanine, pyoluteorin and 2,4-diacetyl phloroglucinol and hydrolytic enzyme chitinase contribute to suppression of *C. falcatum*, induced resistance and growth promotion in sugarcane (36,39,43).

MOLECULAR BASIS OF RED ROT RESISTANCE IN SUGARCANE

Plants under are constant threat to infection by pathogens armed with a diverge array of effector molecules to colonize their host. Plants have in turn evolved a sophisticated detection and response systems that decipher pathogen signals and induce appropriate defence. Recent genetic analysis involving plant mutants defective in resistance response to the invading pathogens has revealed a number of distinct, but interconnecting, signalling networks that are under both positive and negative regulatory control. These pathways operate atleast partly through the action of small signalling molecules such as salicylate, jasmonate and ethylene. The interplay of signals probably allows the plant to fine-tune defense responses in both local and systemic tissue. AT SBI, we have taken up both genomic and proteomic studies to understand the host-pathogen interaction of sugarcane-*C. falcatum* at molecular level. In genomics, we have employed reverse transcriptase - polymerase chain reaction (RT-PCR), differential display RT-PCR, reverse northern and rapid amplification of cDNA ends (RACE) to identify and characterize the resistance/defense genes involved in red rot resistance.

Molecular interactions of sugarcane-*C. falcatum*

Plants are able to recognize fungal pathogens by their secreted products, referred to as 'elicitors'. These elicitor molecules are signal molecules and elicit synthesis of phytoalexins, phenolics, lignin, PR-proteins, hydroxyl-proline rich glycoproteins

and other defense-related metabolites in host plant and thus mediates pathogen recognition. Defence responses are induced upon perception of either specific or non-specific elicitors (1). Specific elicitors are products of pathogen avirulence genes and are hypothesized to be recognized by the products of corresponding pathogen race-specific resistant genes. Detailed studies were carried out to purify and characterize elicitors from *C. falcatum* and to study their role in recognition of *C. falcatum* by sugarcane.

A high molecular weight elicitor was isolated from the mycelial walls of *C. falcatum* and partially purified by gel filtration. The elicitor is characterized as a glycoprotein and the activity of elicitor resides in the carbohydrate moiety. The partially purified elicitor induced the accumulation of phenolics and the activities of phenylalanine ammonia-lyase (PAL) and peroxidase (POX) in both sugarcane leaves and suspension-cultured cells. Sugarcane cells in culture responded to *C. falcatum* elicitor in a manner similar to sugarcane leaves (9). Similarly, induction of hydrogen peroxide (H_2O_2), reactive oxygen species (O_2^-), lipoxygenase, lipid peroxidation, super oxide dismutase (SOD) and catalase was also observed in cell suspension cultures of sugarcane primed with the fungal elicitor. A rapid outburst and a spurt in the generation of active oxygen species especially H_2O_2 was observed indicating an early molecular event in recognition of the pathogen by the host cells. However, higher levels of the suppressor enzymes viz., catalase and superoxide dismutase were found to be maintained throughout in the cells of sugarcane suspension cultures without any elicitor treatment (10). When elicitor isolated from *C. falcatum* was compared with *C. lindemuthianum* - a non-pathogen elicitor, differential induction of POX isoforms in suspension-cultured cells of sugarcane cv. CoC 671 was found (11).

For studying the defense gene activation in sugarcane, the intact plant-pathogen system may not be ideal because the time course of pathogen infection along with the concomitant accumulation of host defense could not be monitored precisely. Cell cultures and elicitors instead of whole plants and live pathogens respectively are found to be suitable models to study the defense gene activation in bean, rice and in sugarcane because of their high degree of reproducibility and rapid experimental cycles. Further, the homogenous cell suspension is uniformly

exposed to the elicitor preparation and hence the response of cells is relatively uniform. Preliminary results of the study conducted at SBI has provided insight into the mechanisms regulating the pathogen recognition at the interface which facilitates further elaboration of inducible defense response against *C. falcatum* in sugarcane (11,12). These studies confirmed that the elicitor molecules from *C. falcatum* are responsible for specific recognition of the pathogen by the host and resistance is determined by the rapidity of the downward signalling of defence pathway. Probably variation in initiation of signalling process between the resistant and susceptible genotype determines the pathogen colonization and disease development *vis a vis* host resistance.

GENOMIC STUDIES IN DEFENCE/RESISTANCE GENE EXPRESSION

To understand genome complexity of sugarcane, a large scale expressed sequence tag (EST) programme known as 'SUCEST' was taken up recently in Brazil. More than 2,60,000 cDNA (complimentary DNA) clones were partially sequenced from 26 standard cDNA Libraries generated from different sugarcane tissues. They annotated 43,141 assembled sequences and found 50% of the putative identified sugarcane genes coding for protein metabolism, cellular communication/signal transduction, bioenergetics and stress responses. Eighty SUCEST sugarcane assembled sequences (SASs) encoded proteins (24) with clear similarity to the nucleotide-binding adaptor shared by APAF-1, R proteins, and CED-4 (NB-ARC) domain was found which is characteristic of one of the major classes of disease resistance genes (R genes). The database contained more than 200 SASs encoding WRKY transcription factor domains, which have been implicated in the defense gene regulation in plants. They also found other genes related to defense responses like chitinases, ß-1,3-glucanases, chalcone syntheses, chalcone isomerases, isoflavone reductases, hydroxylproline rich glycoproteins, proline-rich proteins, catalases, superoxide dismutases etc. have been putative orthologs in sugarcane, which indicates a high conservation of defense strategies among plants. The wealth of information generated in the SUCEST database promises exciting prospects for the scientists involved in sugarcane improvement and other crops. Many transcripts including the disease resistance

and defense responses-related ESTs will be a basic resource for the understanding of the biology of this complex polyploidy plant. This information may facilitate in identifying gene(s) involved in disease resistance in sugarcane.

We have carried out detailed studies on identifying specific transcripts induced during host-pathogen interaction to identify candidate genes involved in red rot resistance. After pathogen inoculation, total RNA was isolated from sugarcane stalk tissues and reverse transcribed to cDNA for expression analysis. The level of expression and the interval at which a particular gene expression etc, were analyzed using the custom made primers. The following transcripts R30, chitinase, metallothionein, receptor protein kinase (RPK) and reversibly glycosylated protein (RGP) were differentially expressed in resistant and susceptible varieties and these clones shared sequence similarity with disease resistance genes in other crops (33). The results also showed an early induction of defense/resistant gene(s) in the resistant variety and in case of susceptible variety, the induction was delayed significantly (Fig. 5). In further studies, they employed differential display (DD)-RT-PCR a powerful technique to identify more number of transcripts involved in pathogen recognition, signal transduction and plant defense in sugarcane. Differential display technique revealed ~450 transcripts to be differentially expressed upon pathogen inoculation in sugarcane. About 202 transcripts were selected for their down-regulation and 243 transcripts were selected for their up-regulation upon pathogen inoculation. After homology search, the expressed sequence tags (ESTs) with a match in the databases (both characterized and uncharacterized) were categorized into eight groups primarily based on putative function. The total up-regulated and down-regulated transcripts were 63% and 37% respectively, indicating a higher percentage of transcripts that seems to be induced in response to pathogen inoculation. Among the known proteins, the signal transduction group represented the highest percentage of up-regulated sequences, followed by protein synthesis and storage, general metabolism, transport, defense, cell structure/growth/division, transcription/post transcription and bioenergetics. The important transcripts identified include 14-3-3-like protein, Senescence-associated protein DH, xylanase inhibitor protein 1 precursor, Putative chitinase, Leucine Rich Repeat family protein, F-box

domain containing protein, UMP/CMP kinase-a and Putative hydroxyproline-rich glycoprotein. Similarly, DD-RT-PCR was conducted with sugarcane cell lines treated with an elicitor molecule identified from red rot pathogen and similar transcripts were found to be differentially regulated upon elicitor treatment. Further northern and reverse northern blot analyses using radioactive α [^{32}P] dCTP confirmed differential expression of potential DD transcripts. This approach would be more useful to identify the potential transcripts during molecular interaction between the host and the pathogen.

SUBTRACTIVE LIBRARIES

DD-RT-PCR studies clearly established differential expression of defence related transcripts in cane tissues as well as suspension cells in response to red rot fungus infection and Cf-elicitor treatment, respectively. Further studies were carried out to construct cDNA libraries in the resistant cv Co 93009, 12h and 72h after pathogen infection. In suppression subtractive hybridization (SSH) only the differentially expressed transcripts in two populations of comparison are subtracted, amplified and cloned. In the study, RNA population from the resistant cv Co 93009 was used as tester and the susceptible cv CoC 671, was used as the driver. Also injured and uninjured controls were maintained. A total of 700 subtracted transcripts encoding different functional categories were obtained and they gave new information concerning disease resistance in sugarcane. The early (12 hr) and late (72 hr) response transcripts were grouped into several functional categories as signal transduction, defense, energy metabolism, carbohydrate metabolism, cell wall strengthening transcripts and transcription factors. The early response library transcripts identified include the following: lipoxygenase, involved in signal transduction, peroxidise a defence response transcript and structural motif transcripts helix loop helix protein and transducin WD-40 repeat protein. Further, a group of cytokine members like thylakoid formation protein and actin related proteins were also induced. Apart from this, acyl carrier protein involved in fatty acid metabolism was also induced in the resistant cultivar. The late response transcripts include the following: signal transduction transcripts of ACC oxidase and ACC synthase, involved in ethylene biosynthesis pathway and

serine/threonine kinase, which signals defense response in plants. The well-documented defense responsive, antifungal genes like chitinase and ß-glucanase were found to be expressed in the resistant cultivar. Adenosine kinase involved in viral defense was also expressed in the resistant cultivar. Transcripts associated with energy metabolism identified include mitochondrial alternative oxidase, uncoupling protein and mitochondrial alternative oxidase. The differentially expressed transcripts involved in carbohydrate metabolism were sucrose transporter, xylosyl transferase and 6- phospho fructo kinase. This is a new information on sugar metabolism and disease resistance in sugarcane. Cell wall strengthening gene transcripts included pectin methyl transferase precursor, xyloglucan endo-trans glycosylase and cellulose synthase. In the study, transcripts of transcription factors like leucine zipper, MYB, ethylene responsive and LIM involved in lignin biosynthesis were also identified as differentially expressed. It was noted that the transcripts involved in protein structure folding and fatty acid metabolism were induced in the early response where as, transcripts associated with sugar metabolism and more of signal transduction were observed in the late response. We have generated detailed information on differential expression of transcripts responsive to red rot infection in sugarcane which are new to sugarcane and further studies are in progress to cluster the transcripts with their functional groups and to validate their functions. Eighty five clusters of expressed gene tags (ESTs) that preferentially express upon *C. falcatum* infection have been identified, which were previously unreported (4). By real time RT-PCR profiling of selected EST clusters, they identified several sugarcane clusters that show differential expression in response to biotic and abiotic stress conditions. In addition to six resistant gene analogues, (RGAs), a full-length R-gene (TIR-NBS-LRR) termed *SNLR* have been isolated (45) from NCo 376, a smut resistant variety in mainland China and its expression profile have been charcterized by real-time RT-PCR,in response to treatments with *Sporisorium scitaminea* causing smut, salicylic acid and H_2O_2. Further studies in this area of work will be more rewarding and would lead to identifying several candidate genes involved in disease resistance in sugarcane.

PHYTOALEXIN PATHWAY TRANSCRIPTS

Since we have established the role of 3-deoxyanthocyanidin phytoalexins in red rot resistance, the genes involved in the flavanoid biosynthetic pathway like coumarate-4-hydroxylase (C4H), chalcone synthase (CHS), chalcone reductase (CHR), flavanoid 3`-5` hydroxylase (F3'5'H) and flavanoid glycosyl transferase (FGT) were further studied to establish relation between transcript accumulation and differential accumulation of phytoalexins in the resistant and susceptible cultivars. The pathogen inoculation enhanced their expression of C4H especially in resistant variety, which may have positive correlation with their ability to synthesize various downstream compounds like phenolics and flavonoids as reported earlier from various biochemical studies. Further along the phenypropanoid pathway after the conversion of 4-coumaryl CoA to flavone, the flavone and isoflavonoid pathway is initiated. The CHS and CHR are key enzymes of phenyl-propanoid pathway diverting the substrate, naringenin chalcone to the flavanoid and isoflavanoid branches of the phenyl-propanoid pathway that synthesizes the precursor of a large number of secondary metabolites, including pro-anthocyanidins, anthocyanins, flavones, flavonols and isoflavanoid-phytoalexins among others (46). Maximum induction of CHS was found in resistant variety, whereas in susceptible variety this induction was minimal. The F3'5'H transcript was found to be induced upon pathogen inoculation in the leaves of both the varieties and the flavonoid 3'5'-hydroxylase enzyme functions at an important branch point between flavonol and anthocyanin synthesis, as is evident from studies in petunia (*Petunia hybrida*), and potato (*Solanum tuberosum*). Induction of F3'5'H leads to the synthesis of flavanoids, flavanols, flavanones and phenolics. Reports of plant molecular responses to elicitor or pathogenic infections have pinpointed increase in activity of several genes of the phenyl-propanoid pathway leading to the synthesis of phenyl-propanoid metabolites, lignin and flavanoids. The accumulation of phytoalexins in sorghum leaves and mesocotyl tissue after infection with *Colletotrichum sublineolum* (causing anthracnose) was established (2). After penetration of the fungus into the host cell, it was restricted within infected regions by 72 h and an intense reddish pigmentation was observed by 36 h in the resistant cultivar, whereas in the susceptible variety the

phytoalexin appears only by 48 h with proliferation of the pathogen. The HPLC profile of the phytoalexin from the resistant variety showed presence of luteolinidin, 5-methoxyluteolinidin, apigeninidin and the caffeic acid ester (CAE) of arabinosyl 5-O-apigeninidin, while susceptible variety showed presence of only two fractions apigeninidin and CAE of arabinosyl 5-*O*-apigeninidin. Their temporal studies revealed the accumulation of CHS and PR-10 to occur by the 24 h in the resistant variety, while the same occurred only by the 36 h in the susceptible cultivar. Also the level of PR-10 transcript accumulation was lower in the susceptible cultivar than that in the resistant cultivar. Timely expression of the specific transcript at the right place in sufficient quantity is a pre-requisite in the development of resistance in a host system. Our studies have clearly shown the differential pattern of spatial accumulation of transcripts of phenylpropanoid pathway in resistant and susceptible varieties after inoculation of *C. falcatum* in sugarcane.

RAPID AMPLIFICATION OF CDNA ENDS (RACE)

The potential defense related transcripts were selected for RACE analysis to clone the full length cDNAs. Compatible primers were designed at the 3' side using the available partial sequence information and used in the RACE protocol. Using RNA ligase mediated - RACE, we have isolated full length sequences of the following four genes *viz.* 14-3-3 like protein, chitinase, xylanase inhibitor and basal antifungal peptide. After full length isolation of 14-3-3 like protein by RACE-PCR, it has been characterized using bioinformatics tools. The CDD search in the NCBI database revealed the presence of conserved 14-3-3 superfamily domain, 5'UTR (1-86bp), ORF (87-857 bp) and 3'UTR (858-1094 bp) in the full length sequence of 1094 bp**.** The 14-3-3 homologues are known to mediate signal transduction by binding to phosphoserine-containing proteins. They are involved in growth factor signalling and also interact with MEK kinases.

CHARACTERIZATION OF SUGARCANE CHITINASE

Differential expression of sugarcane chitinase in stalk tissues during pathogenesis of *C. falcatum* was established by RT-PCR studies in a set of resistant and susceptible cultivars and the

result was confirmed by reverse northern analysis. In further studies full length of the gene was isolated and bioinformatic analysis was done to identify its functional domains and to predict the three dimensional structure of full length chitinase sequence. Translated sequences revealed the typical characteristics of family 19 glycosyl hydrolase, class I/IV chitinase starting with a signal peptide and ending with a signature domain. Phylogenic study grouped sugarcane chitinase in class IV, based on major deletions in catalytic domain. The close structural template 2DKV (Rice class-I chitinase) was successfully used for the prediction of sugarcane chitinase 3D model. The chitinase gene was cloned in an expression vector and studies are in progress to purify the expressed protein and to assess its biological activities.

Transcriptomic analysis of SAR and *C. falcatum* – responsive transcription factors in sugarcane

Two types of stalk tissue samples viz. systemic acquired resistance (SAR) – responsive tissues of disease susceptible (cv CoC 671) and resistant (Co 93009) and susceptible (cv CoC 671) were selected to perform transcriptomic analysis of SAR mediated resistance and pathogen responsive transcription factors (TFs) involved in sugarcane. Among the 41 TFs screened for differential regulation, 15 of them were found to be up-regulated upon SAR inducer priming and 24 TFs were up-regulated upon pathogen challenge. Upon comparison of SAR inducer priming and in-compatible interaction, 8 TFs were found to be differentially up-regulated in both the cases. Genes representing WRKY family, MYB class of TFs, TLP, NAC and bZIP TFs were screened for their differential response against SAR-priming. Upon SAR inducer treatments, WRKY 40 and 44 were induced at earlier time interval (6 hpi) in BTH and *C. falcatum* elicitor treated samples and also in pathogen challenged samples, but the level of induction was high at a later interval (72 hpi) due to BTH priming. Up-regulation of MYB 78 and 83 were observed in response to BTH-priming. Upon comparison of Co 93009 (incompatible) and CoC 671 (compatible) response to pathogen challenge, most of TLP genes (TLP K and TLP L) were induced at earlier intervals between 0 and 24 hpi in Co 93009; however in CoC 671, these genes were induced only at later intervals between 24 and 48 hpi,, indicating that these TFs might be involved in

activating defense early against pathogen attack in in-compatible interaction and SAR response. Similarly NAC C, D and E were induced at earlier intervals between 6 and 12 hpi in BTH primed tissues, but there was no such induction observed in *Cf* elicitor treated samples, thus indicating that TF regulated gene expression involves multiple signals forming a network of gene activation cascade. Variation in the expression pattern of individual bZIPs at different time intervals was observed. In inoculated control, there was an inconsistent regulation of these genes, induced between 6 and 72 hpi. The overall result indicated the possible role of few of these TFs in regulating SAR induced defense in sugarcane.

Molecular basis of systemic acquired resistance (SAR) mediated resistance

A consistent control of red rot was observed from the treatment of BTH, SA and Cf-elicitor in two different cvs, CoC 671 and CoC 92061 of sugarcane which are highly susceptible to the disease. This study not only explored the potential of SAR-elicitors but also the modes of pre-treatment such as sett treatment, foliar spraying and drenching in marcotted plants and timing of their application in sugarcane. Nevertheless marcotting was found to be effective; it is impractical to apply this method under field conditions. BTH (250 µM), SA (250 µM) and Cf-elicitor (60 µg glc equivalent) have proven to be effective inducers of systemic resistance in sugarcane against red rot. The results of the *in vitro* bioassay and antifungal assays revealed pathogen growth inhibition by metabolites/hydrolytic enzymes from the primed tissues indicating a possible involvement of SAR induced defence-related enzymes in restricting the pathogen growth (15). Possibly antifungal assay of marcotted stalk tissues was the first study to show the direct antifungal effect of primed tissues under *in vitro* conditions against the target pathogen.

The mechanism of induction of systemic resistance conferred by these SAR inducers (BTH, SA and Cf-elicitor) was studied through biochemical studies of defense related metabolites/ enzymes as well as isoenzyme and Western blot analyses of PR-proteins. It was observed that the induced resistance against red rot was correlated with increased activities of peroxidise, polyphenol oxidase, phenylalanine ammonia-lyase, tyrosine

ammonia-lyase, super oxide dismutase, lipoxigenase, H_2O_2 generation, accumulation of phenolics and coumarins. Many of the PR proteins viz., PR1-C, PR-2, PR-3, PR-5 were also found to be induced in SAR primed sugarcane stalk and leaf tissues. Strong correlation was observed between increased activities of PR proteins in the leaf tissues and reduction of disease incidence. Microtitre plate assay gave further evidence for the induction of systemic resistance from the treatment with chemical activators in sugarcane.

BTH, SA and Cf-elicitor induced changes in the expression of key enzymes involved in the phenylpropanoid pathway was analyzed by reverse transcription (RT)-PCR approach. The results evidenced that induced resistance in sugarcane is largely correlated with the phenylpropanoid pathway enzymes which lead to the synthesis of coumarins, lignins, cell wall bound phenolics, flavanoids, flavanols, flavanones, isoflavanones and isoflavanols. RT-PCR work with the genomic scanning of the induced transcripts for R-genes and other defense-related genes revealed that SAR mediated resistance in sugarcane is largely correlated with R-genes, DIR, BAF and defense related enzymes which are involved in plant defense mechanism and pathogen degradation processes. Future work underlies on elucidating specific signalling pathways and identification of key molecules that could be modulated for inducing systemic resistance in sugarcane. Such approaches hold promise in future to achieve a tangible red rot management strategy in sugarcane and to understand disease resistance mechanism in sugarcane.

DEFENCE PROTEOMICS STUDIES IN SUGARCANE

The term proteome refers to the complete set of proteins that are specified by the genome, and analogous to genomics, proteomics describes the study and characterization of this complete set of proteins present in a cell, organ or organism at a given time. Genome-level studies (genomics) reveal or suggest what could theoretically happen, whereas the proteome-level investigation (proteomics) provides insights into the actual players involved in mediating specific cellular processes. In addition, the study of proteins introduces another level of complexity at the level of the post-translational modification (PTM) and the biological relevance of such modifications. These changes in PTM

during the growth and development of organisms (including plants) or in response to stress (including disease) cannot be deduced from studies investigating genome sequences and/or transcript abundance. Such changes can only be deciphered through proteomics and it is a powerful tool in understanding which proteins are present in particular tissue under given conditions and it is one of the fastest growing areas of biological research. In addition to the enzymes, transport and regulatory proteins, many proteins contribute in cascades of reactions leading to the metabolites involved in disease resistance. This makes the proteome an essential target for studying metabolic pathways.

Information on the clearly identified proteins during host-pathogen interactions and defense signaling in sugarcane is lacking to understand disease resistance mechanism(s). Hence we have standardized a protocol for extraction of proteins for 2-DE from the rigid, fibrous sugarcane stalk tissues to identify specific proteins involved in resistance to red rot in sugarcane tissues for the proteome analysis. Among the five methods tested, 2D cleanup-phenol method was found to be the most suitable for producing high number of good quality spots and reproducibility. Thirty protein spots commonly present in three methods were selected and subjected to *eLD*-IT-TOF-MS/MS analysis and a reference map has been established for sugarcane stalk tissue proteome. This is the first study on sugarcane stalk proteome analysis which possibly will show a new light on unexplored areas of sugarcane proteome research (8).

After establishing stalk specific proteome in sugarcane, detailed studies were conducted on SAR proteomics using BTH as an abiotic elicitor in susceptible cv CoC 671 and defence proteomics in resistant cv Co 93009. The SAR induced proteins were identified by 2-DGE, wherein four protein extraction methods were employed to identify the method specific protein induction in SAR primed tissues. After 2-DGE, the gels were silver stained and differentially expressed spots were picked, digested with trypsin and subjected to MS/MS analysis. The results of peptide mass fingerprinting was analyzed using MASCOT protein identification software. Comparative 2-D gels analysis identified 19 highly confident differential protein spots in BTH treatment over control. The differentially expressed spots were identified as COMT, profilin 4, Cu/Zn SOD, UDP glucose

DHase, isocitrate lyase, GAPDH, Os05g0501300 protein, IN2-1 protein, Nucleoside diphosphate kinase 1, unknown protein, PAL, Quinone reductase 2, Glutathione transferase 9 etc. Detailed defence proteomics in sugarcane revealed that the number of protein spots was found to be higher in the resistant cultivar cv Co 93009 after 12 h of pathogen challenge, whereas the inoculated susceptible cv CoC 671 had the lowest number of protein spots. More than 250 protein spots that were detected in stalk tissues by proteomic analysis showed reproducible abundance within replications. Approximately 50 protein spots were additionally induced in the resistant cultivar upon pathogen inoculation, whereas ~ 24 proteins have disappeared in susceptible cultivar. These studies have established that proteome analysis has the potential to provide significant insights into the molecular events that occur during sugarcane–*C. falcatum* interactions and this is the first attempt to standardize proteome analysis and to identify proteins involved in red rot resistance in sugarcane (32). Further studies using peptide mass finger printing would characterize the up/down regulated proteins and their role(s) in red rot resistance will be established.

CONCLUSION

Overall, the studies conducted at SBI revealed definite role of 3-deoxyanthocyanidin phytoalexins and certain PR-proteins in red rot resistance. Although they are not the primary determinants of disease resistance they have been well established as potential antifungal weapons used by the plants to arrest the invading pathogen. Ongoing studies in our lab on identifying molecular basis of red rot resistance would reveal clear understanding of the molecular communication between the host and the pathogen that ultimately decides disease resistance. The newly identified transcripts will be good candidates for in-depth analysis to elucidate *C. falcatum*-responsive pathways in sugarcane and facilitate genetic manipulation to tailor this crop for tolerance to red rot and other diseases. Also disease resistance genes identified in our studies that showed differential regulation can provide preferred targets for breeding or to engineer durable disease resistance in sugarcane. Creating subtractive libraries to identify more resistance associated genes are in progress and detailed analyses of gene functions of the newly identified genes would help

better understanding of the host resistance mechanisms underlying the response of sugarcane plant to different biotic and abiotic stresses.

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