Identification of differentially expressed transcripts in sugarcane upon *Colletotrichum falcatum* infection by SSH

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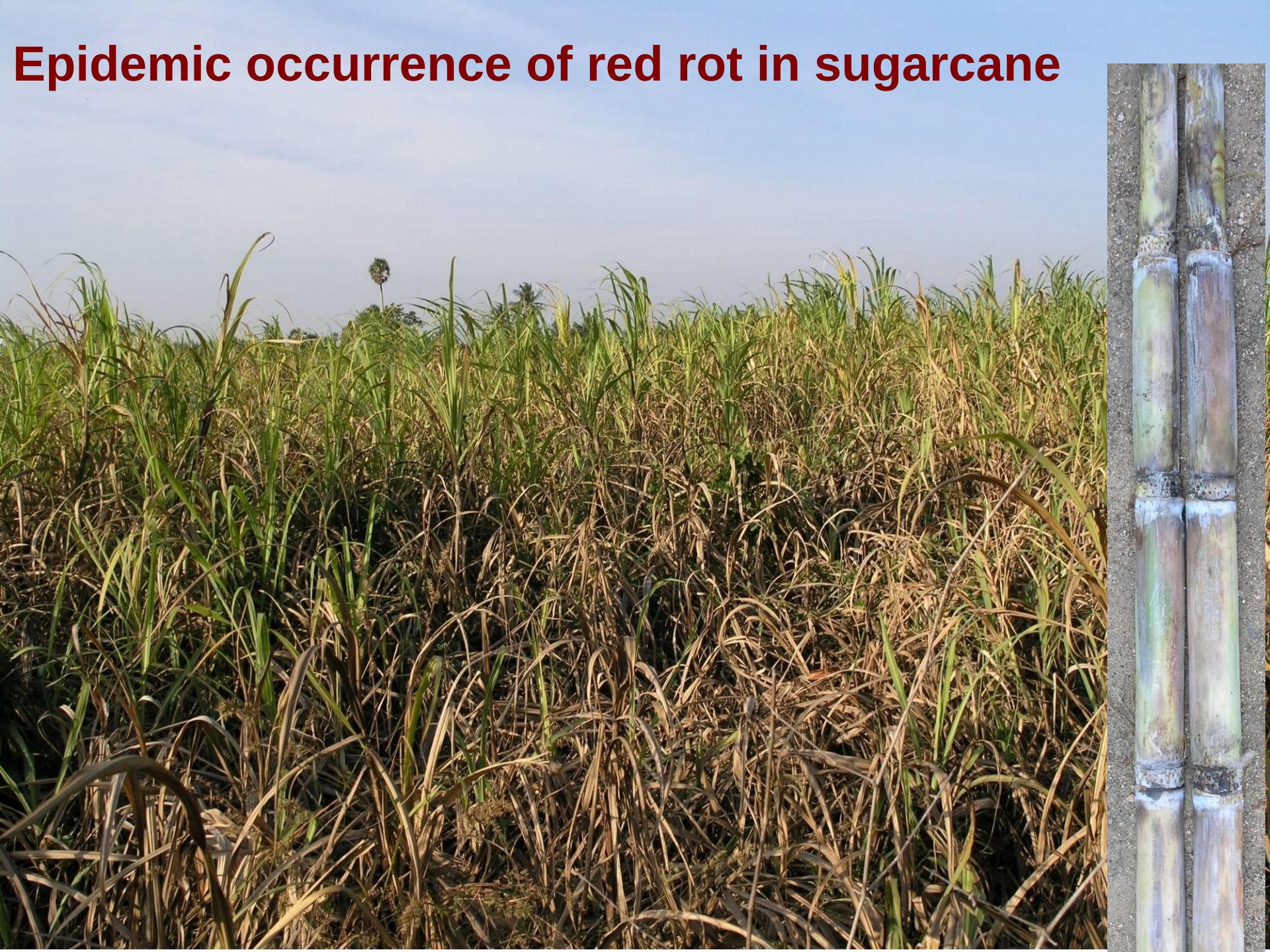
Red rot of sugarcane

- Pathogen - *Colletotrichum falcatum* Went
- The most destructive disease of sugarcane in India
- Infection of stalks in sugarcane, which are economically important.
- Elimination of elite commercial varieties like Co 419, Co 658, Co 997, Co 1148, Co 62175, Co 6304, CoC 671, CoJ 64, CoS 8436, CoSe 95422 etc.
- Regular disease epidemics recorded during the last 100 years .
- Yield loss of upto 100%.



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Epidemic occurrence of red rot in sugarcane



Constraints in varietal development

- Frequent breakdown of varieties to red rot
- Inheritance of red rot resistance in sugarcane – not known.
- Limited information on mechanism of resistance.



Screening and categorization of red rot resistance in sugarcane



Resistant



Moderately Resistant



Moderately Susceptible



Susceptible



Highly Susceptible

Red rot - Reaction of resistant and susceptible clones



Mechanism of resistance

Mechanical

Biochemical

Phenyl propanoid enzymes

- **Phenylalanine ammonia-lyase, Peroxidase, 4-Coumarate CoA ligase**

Phytoalexins

- **3-deoxyanthocyanidins**

Pathogenesis-related proteins

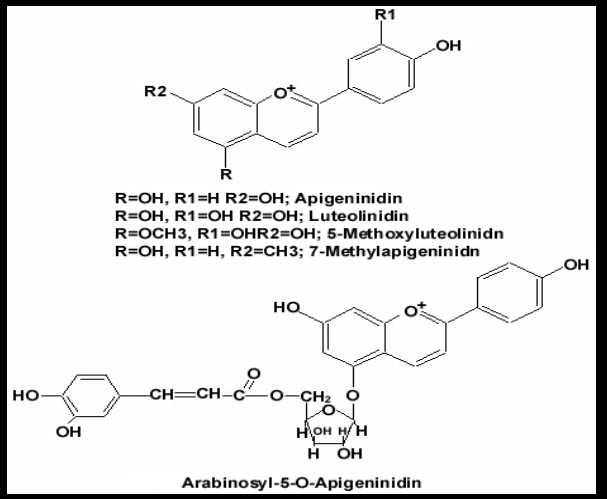
- **Chitinase, beta-1,3–glucanase and Thaumatin-like proteins**

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Mechanism of resistance

Role of 3-deoxyanthocyanidin phytoalexins

Luteolinidin, apigeninidin and caffeic acid ester of 5-0-apigeninidin





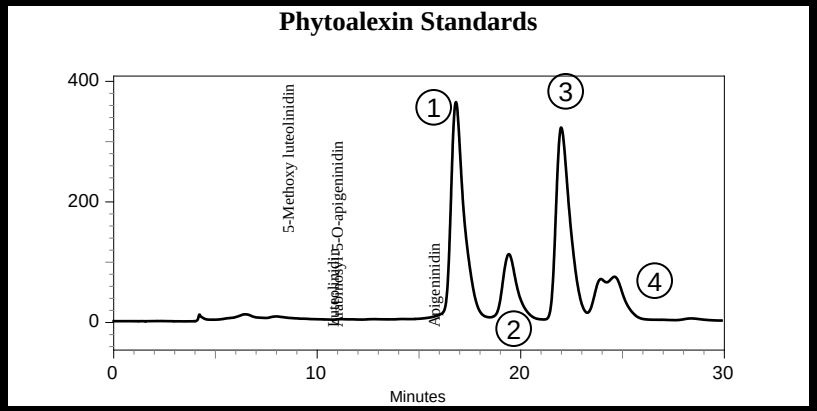
Phytoalexin accumulation in resistant variety



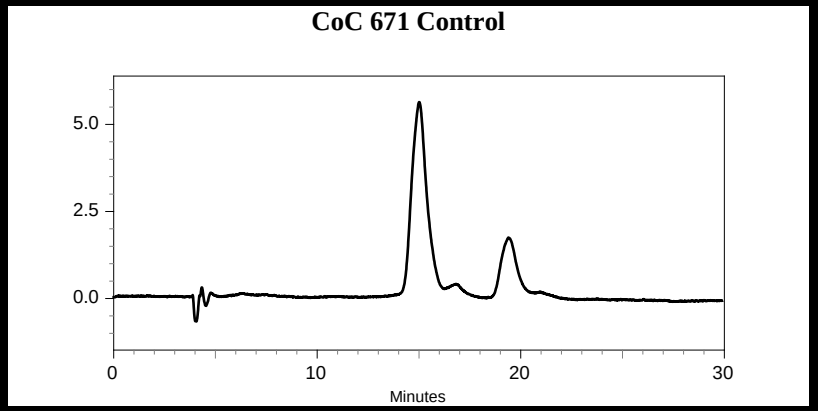
No Phytoalexin accumulation in susceptible variety

RV

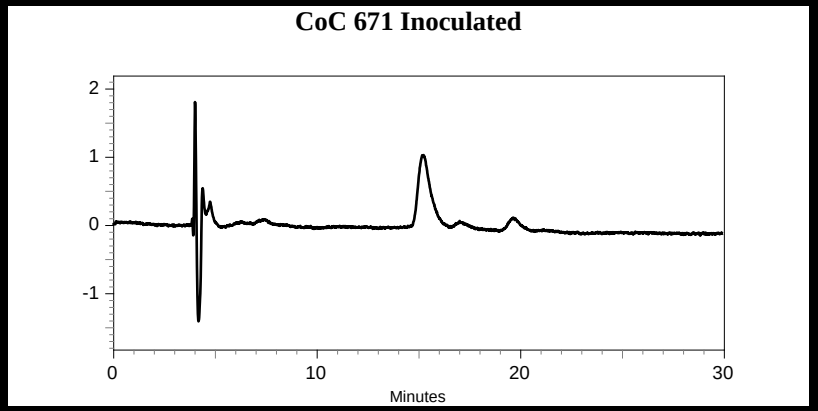
HPLC PROFILE

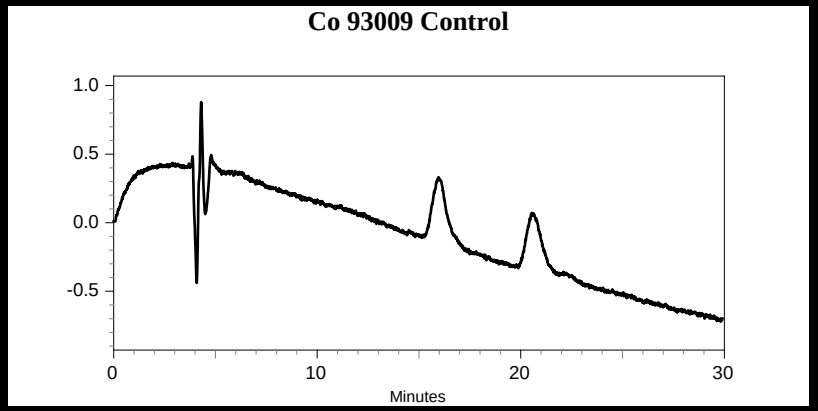


① Luteolinidin
② 5-methoxy Luteolinidin
③ Apigeninidin
④ Arabinosyl-5-O Apigeninidin

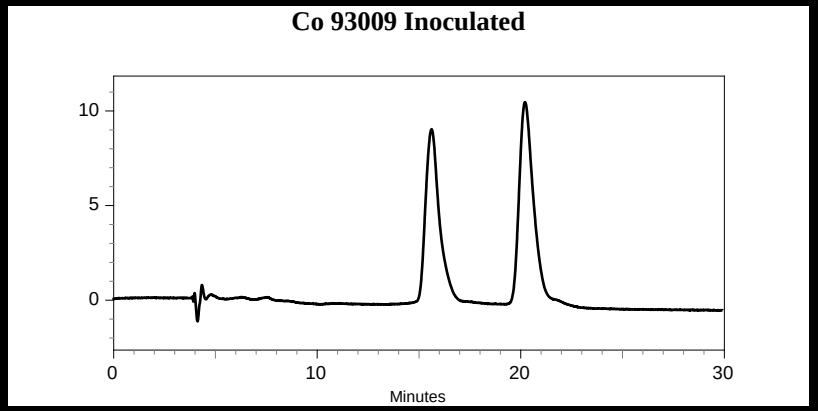


S





R

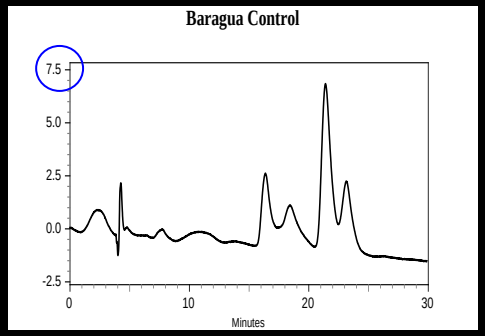


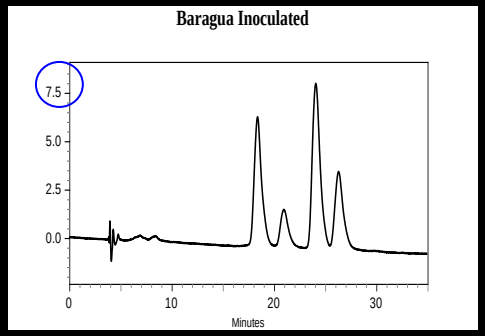
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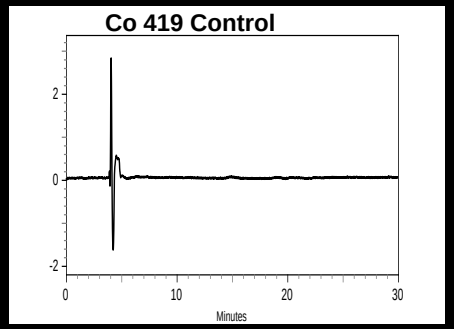
Differential accumulation of 3-deoxyanthocyanidin phytoalexins in sugarcane in response to *C. falcatum* challenge

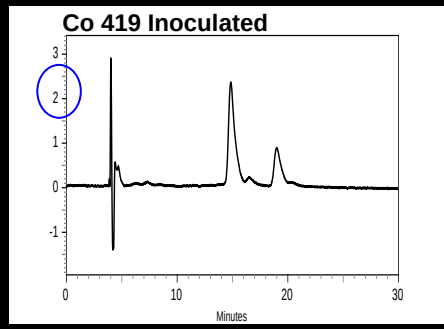
Resistant varieties

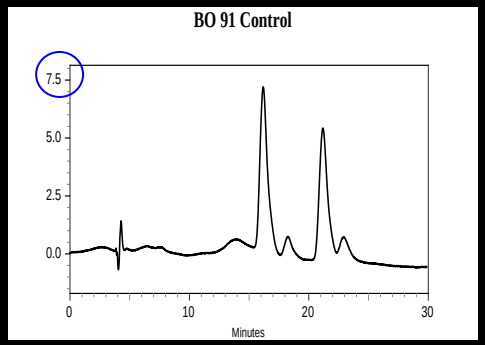
Susceptible varieties

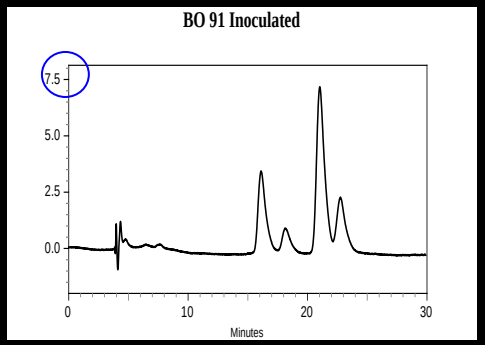


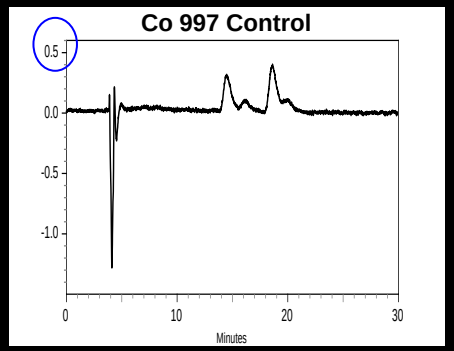


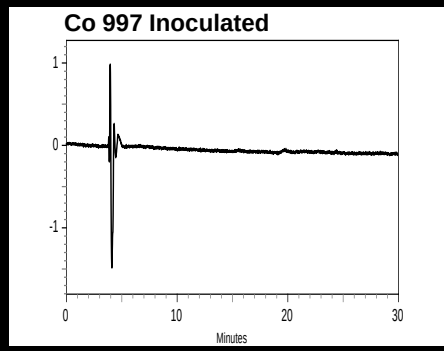








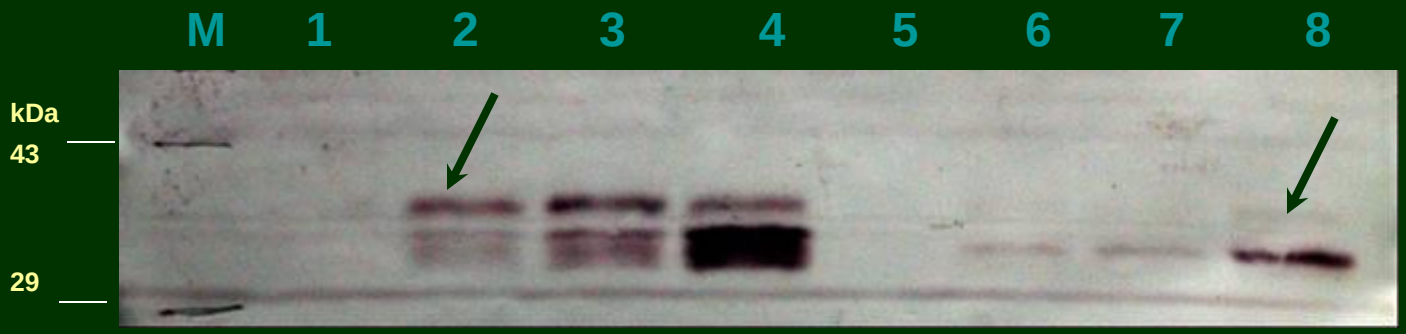




Resistant

Susceptible

Differential induction of chitinase in resistant (Co 93009) and susceptible (CoC 671) varieties in response to pathogen inoculation



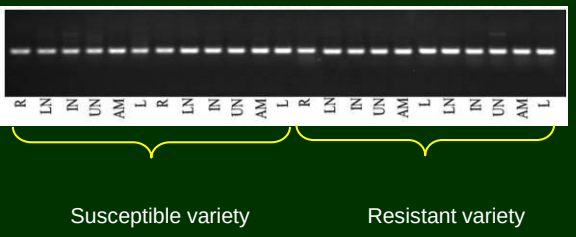
M. Protein markers

Resistant 1. 0-hr 2. 18-hr 3. 24-hr 4. 42- hr

Susceptible 5. 0-hr 6. 18- hr 7. 24-hr 8. 42-hr

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Spatial expression analysis of transcripts by semi-quantitative RT-PCR - Chalcone reductase



First leaf (L)

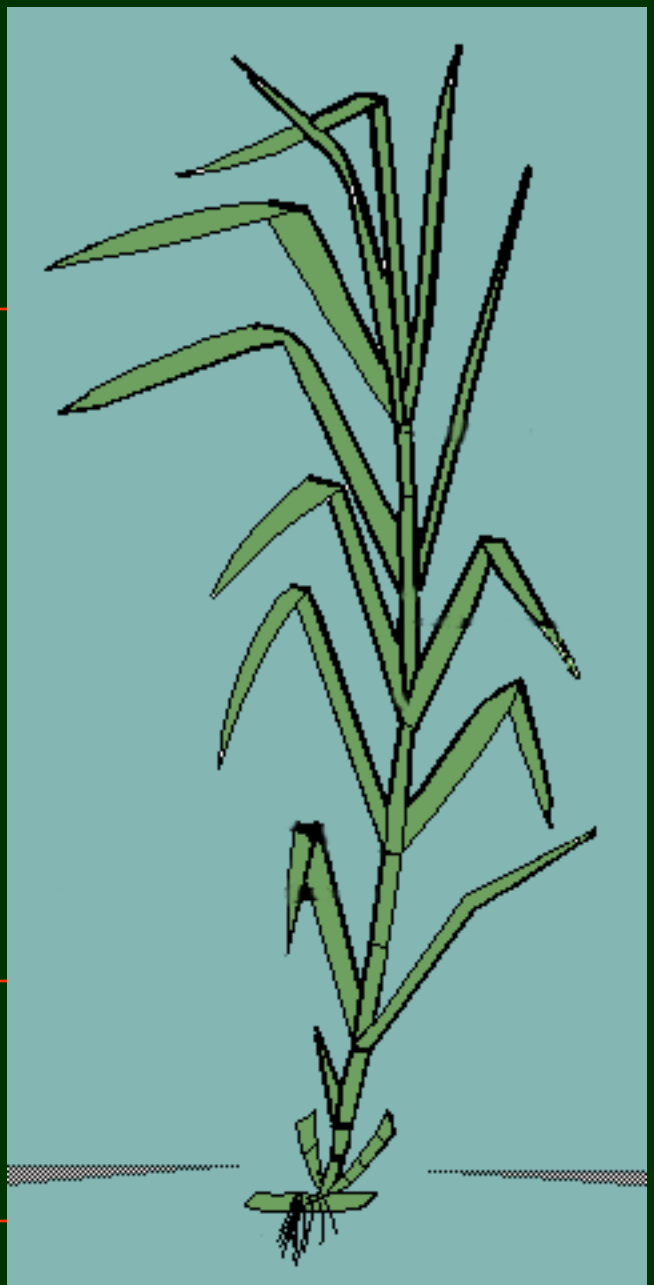
Apical meristem (AM)

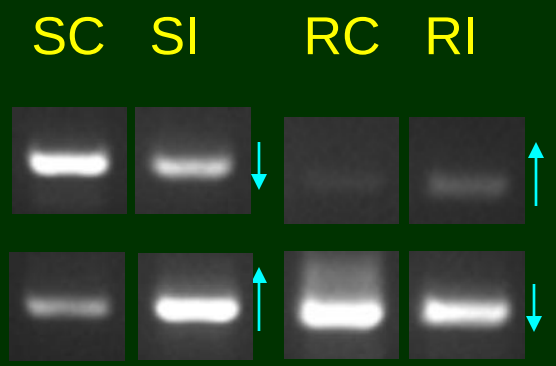
Upper internode (UN)

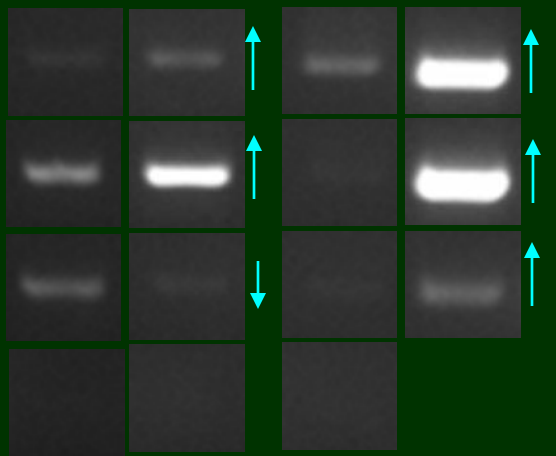
Inoculated internode (IN)

Lower internode (LN)

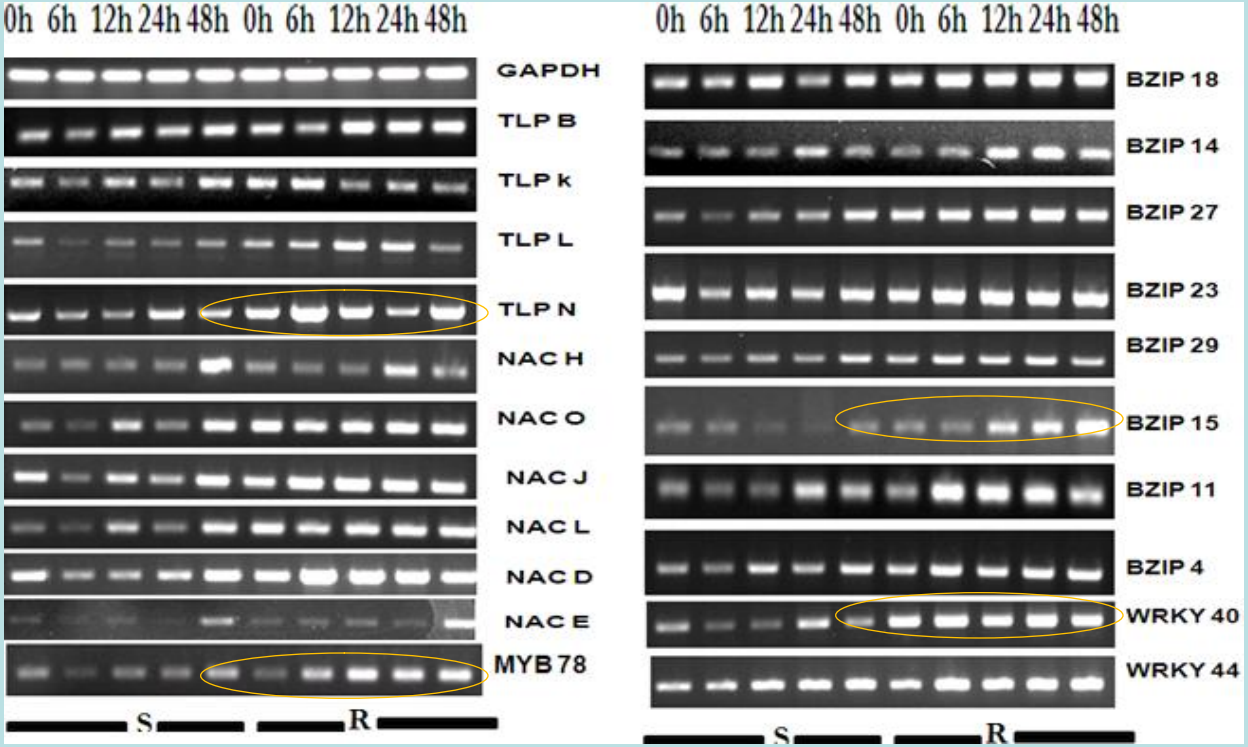
Root (R)







Expression of defence related transcription factors after pathogen infection in different time intervals

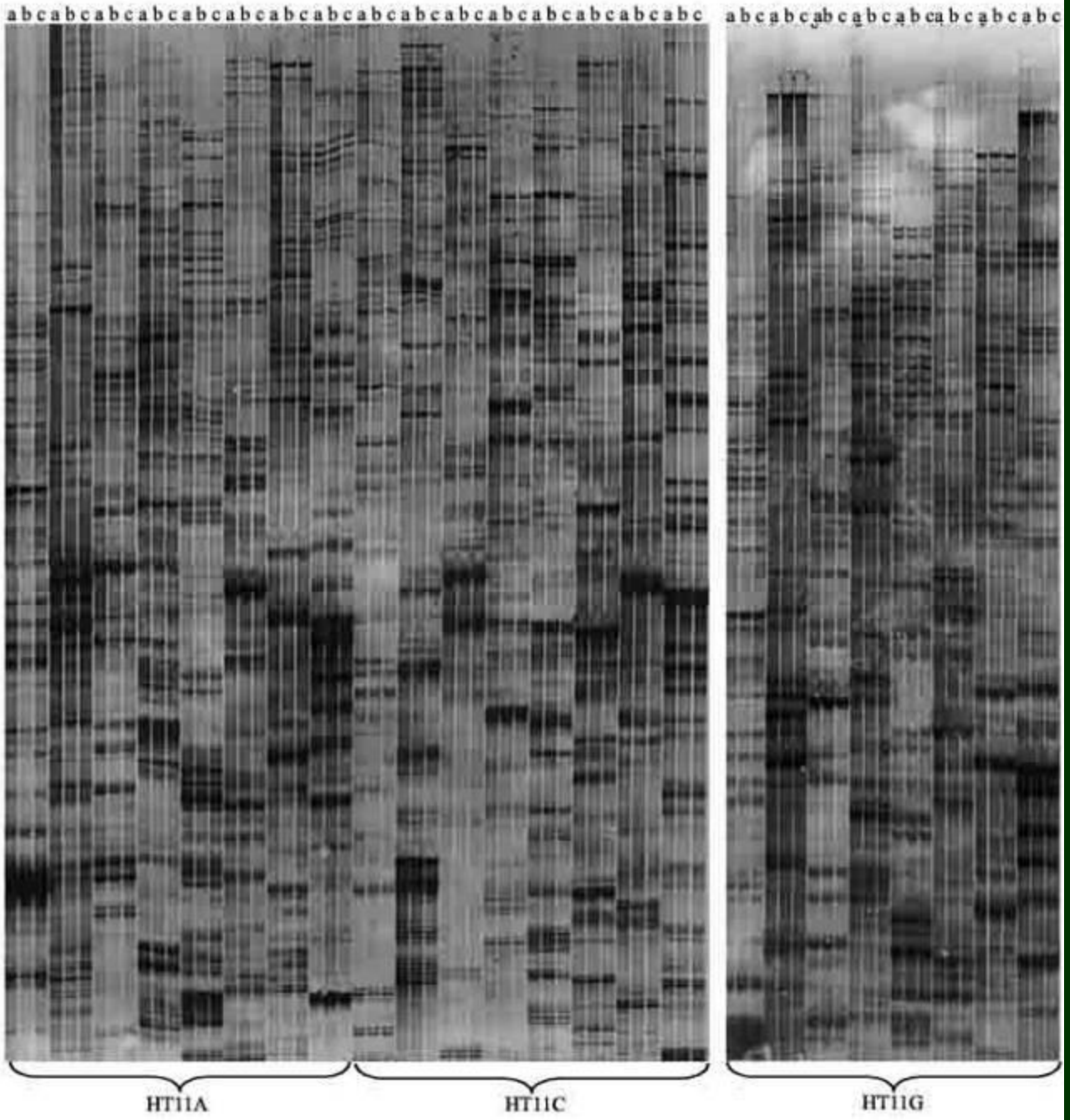


S- red rot susceptible cv CoC 671; R- red rot resistant cv Co 93009 varieties of sugarcane

Defence related transcription factors in sugarcane

Transcription factors	TFs induced during SAR and incompatible (R) interactions
MYB 78	✓
WRKY 40	✓
WRKY 44	✓
TLP K	✓
TLP N	✓
BZIP 4	✓
BZIP 18	✓
NAC D	✓
BZIP 15	
BZIP 29	
TLP B	
NAC E	

Differential display



(* a-Control, b-30min (elicitor treated), c-3hr (elicitor treated)

Autoradiography image of differential display pattern of sugarcane variety Co 93009 (elicitor treated cell suspension) amplified using 3 anchor primer H-T11(A/G/C) and 8 arbitrary 13-mers (HAP-1-8)

Established callus

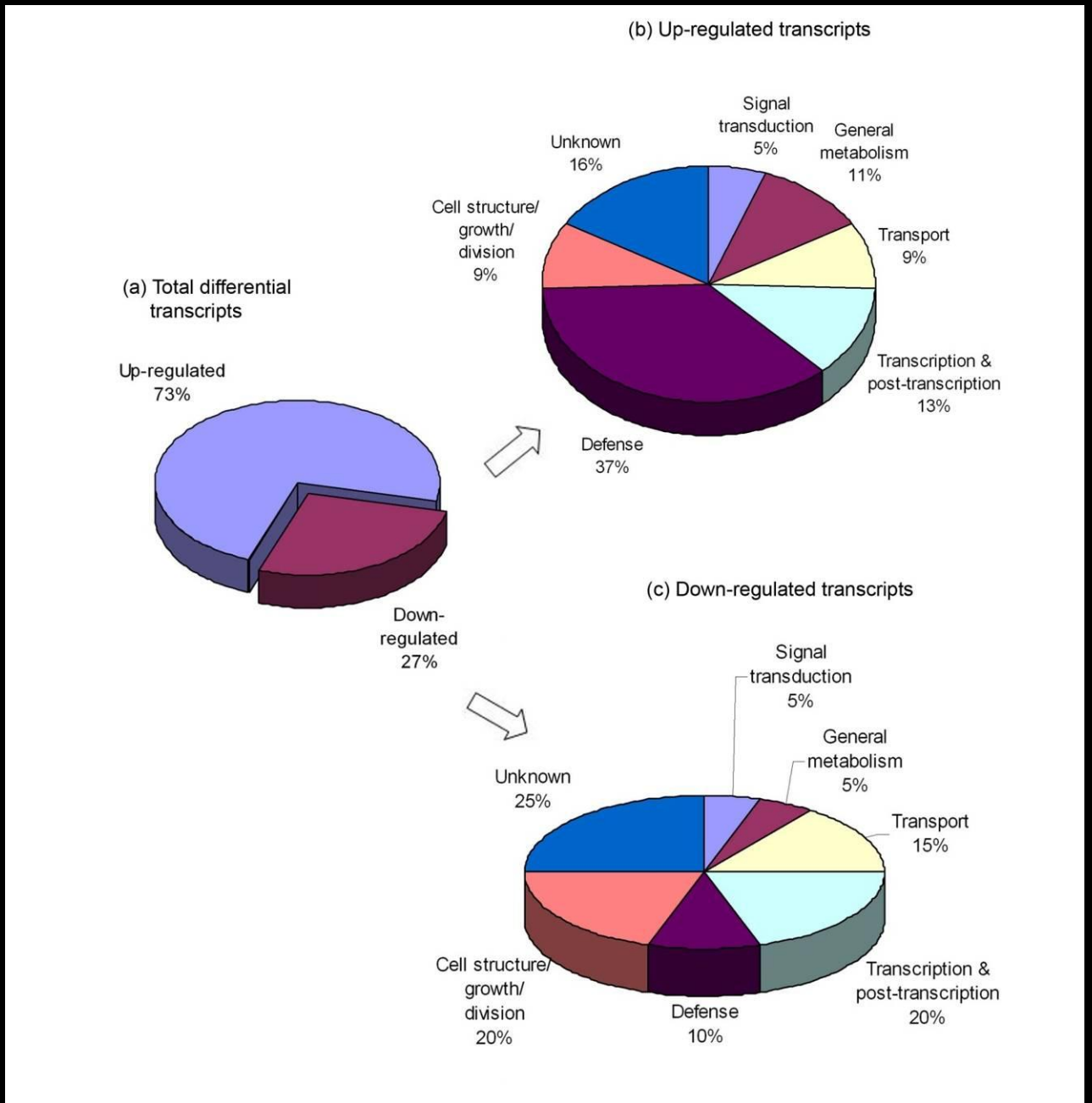


Suspension culture



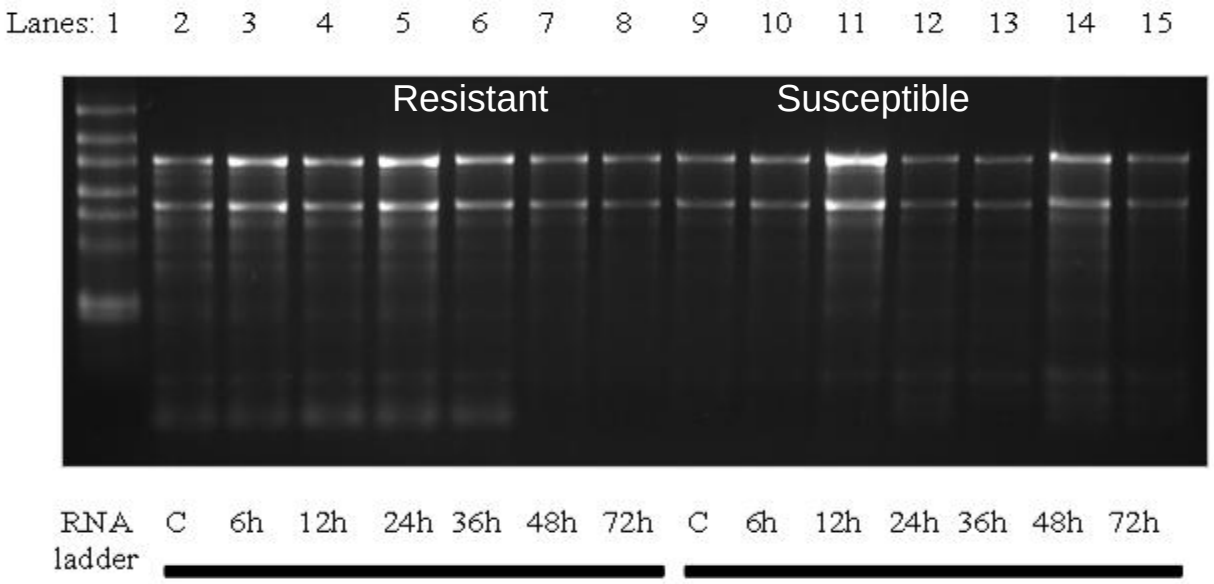
RV

Relative abundance of differential display transcripts identified from *Cf*-elicitor treated sugarcane cell suspensions, categorized based on their putative functions.

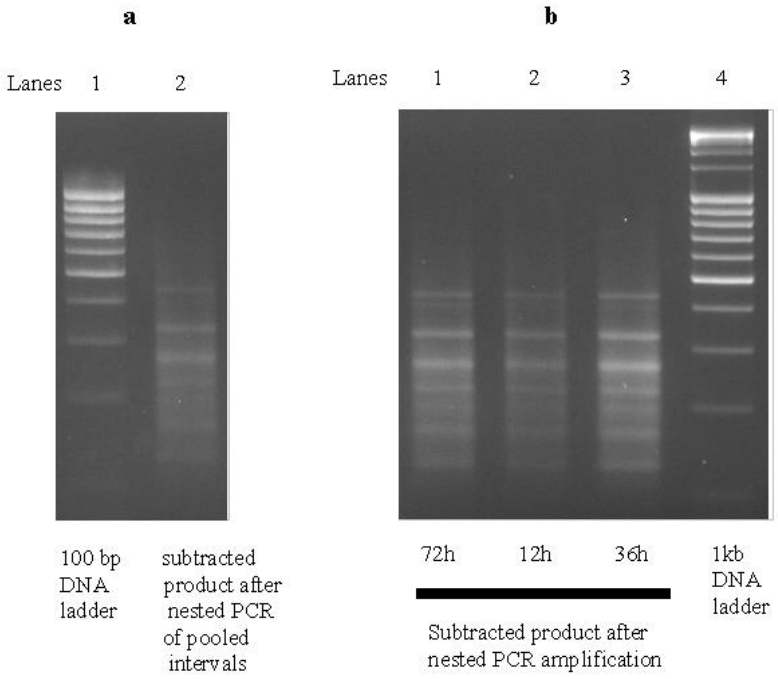


SSH cDNA library construction

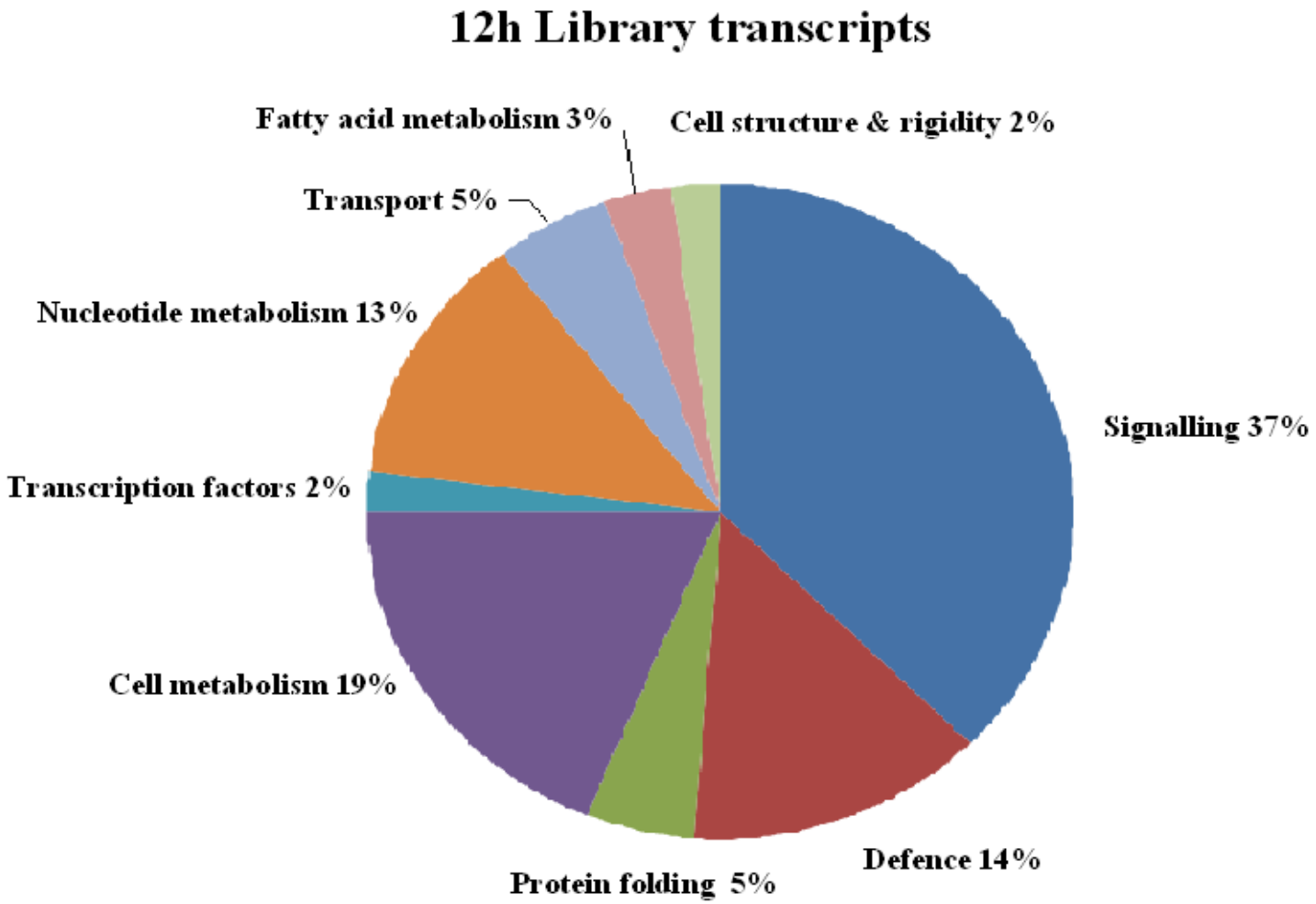
- Three stage specific SSH cDNA libraries were constructed in sugarcane stalk tissue inoculated with *C. falcatum* in resistant (Co 93009) and susceptible (CoC 671) cultivars.
- 3 SSH cDNA libraries were constructed separately from samples harvested at (12, 36 and 72) h after pathogen inoculation representing transcripts at that particular stage/time.
- The susceptible cultivar's cDNA was subtracted from resistant cultivar's cDNA, amplified, cloned and sequenced
- A total of 930 transcripts were obtained from the three libraries; 265 of them were functionally annotatable including multiple copies of transcripts
- Each of them were grouped into functional categories (stage specific)
- Validation of the transcripts by quantitative real time PCR and Northern hybridization are in progress

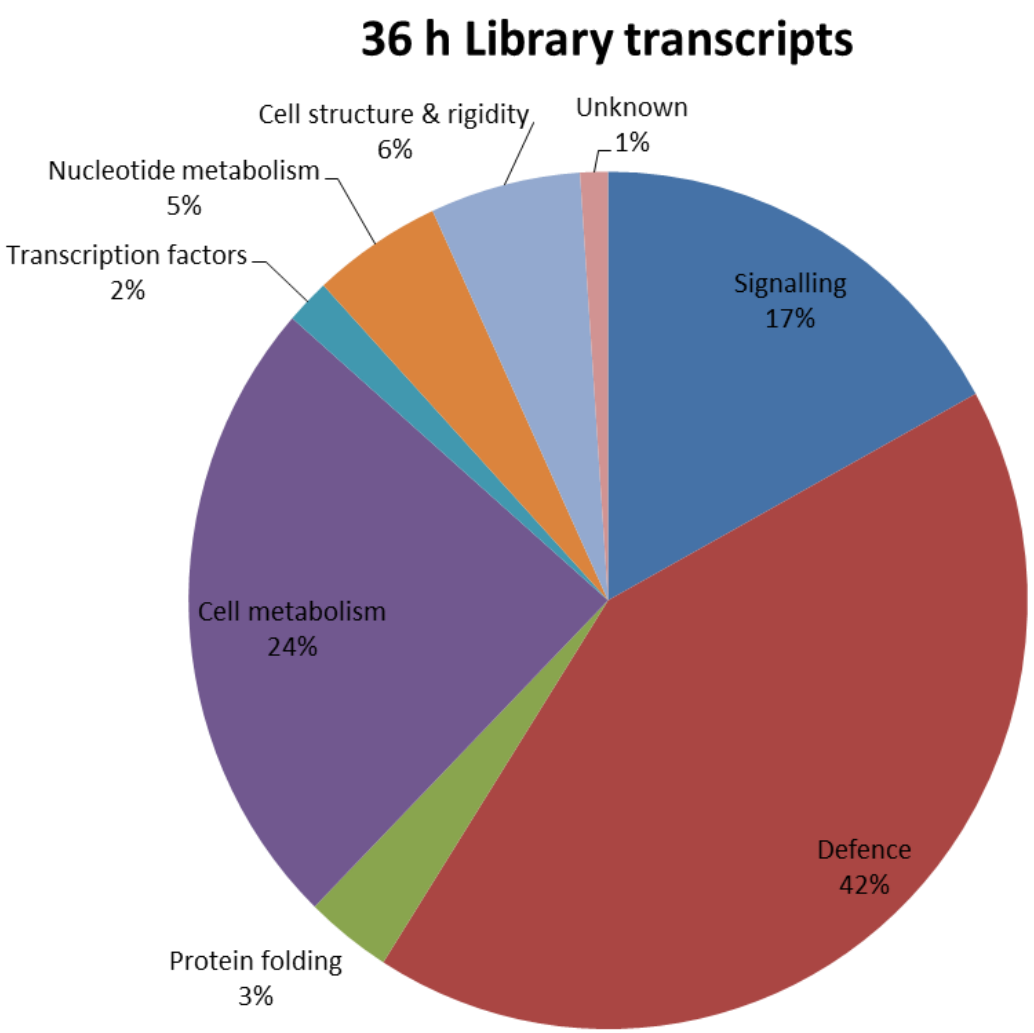


1.5% agarose gel showing prominent RNA bands corresponding to 28S and 18S.



Subtracted PCR products after nested PCR on 2% agarose gels


12h Library transcripts
Fatty acid metabolism 3%
Cell structure & rigidity 2%
Transport 5%
Nucleotide metabolism 13%
Signalling 37%
Transcription factors 2%
Cell metabolism 19%
Defence 14%
Protein folding 5%


36 h Library transcripts
Cell structure & rigidity
6%
Unknown
1%
Nucleotide metabolism
5%
Transcription factors
2%
Signalling
17%
Cell metabolism
24%
Defence
42%
Protein folding
3%

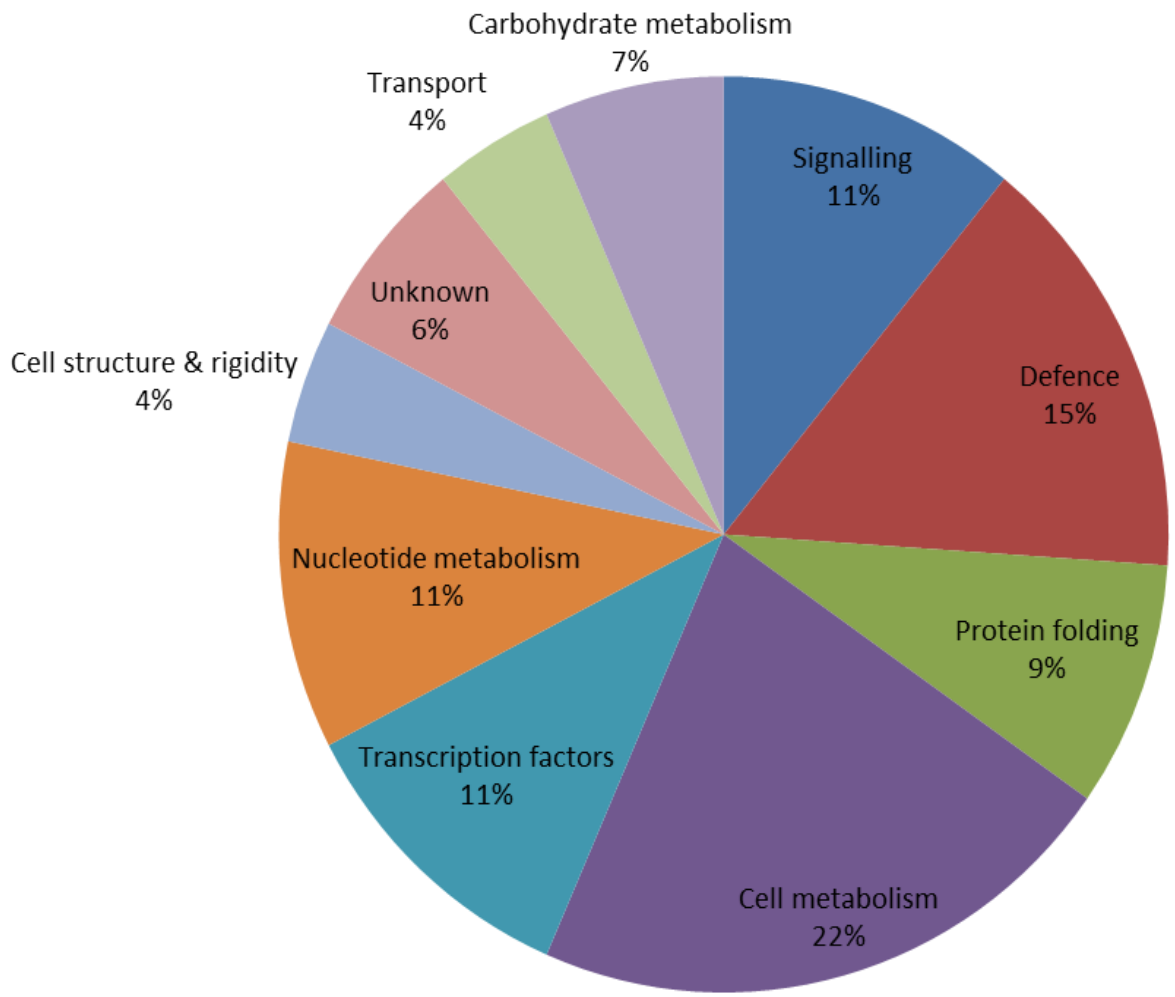
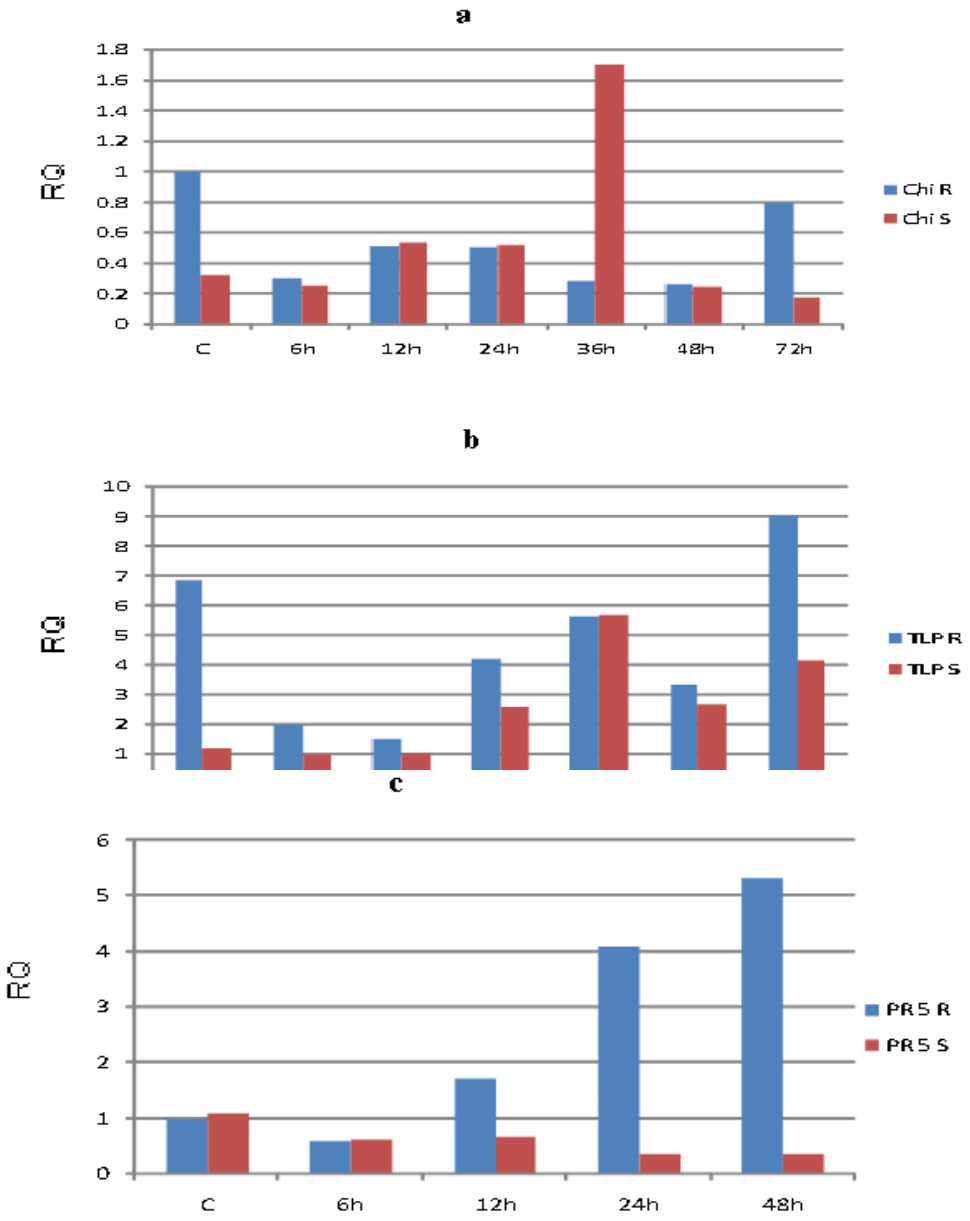

72 h Library Transcripts
Carbohydrate metabolism 7%
Transport 4%
Signalling 11%
Unknown 6%
Cell structure & rigidity 4%
Defence 15%
Nucleotide metabolism 11%
Protein folding 9%
Transcription factors 11%
Cell metabolism 22%

Table representing the percentage of functional groups of transcripts obtained from each library

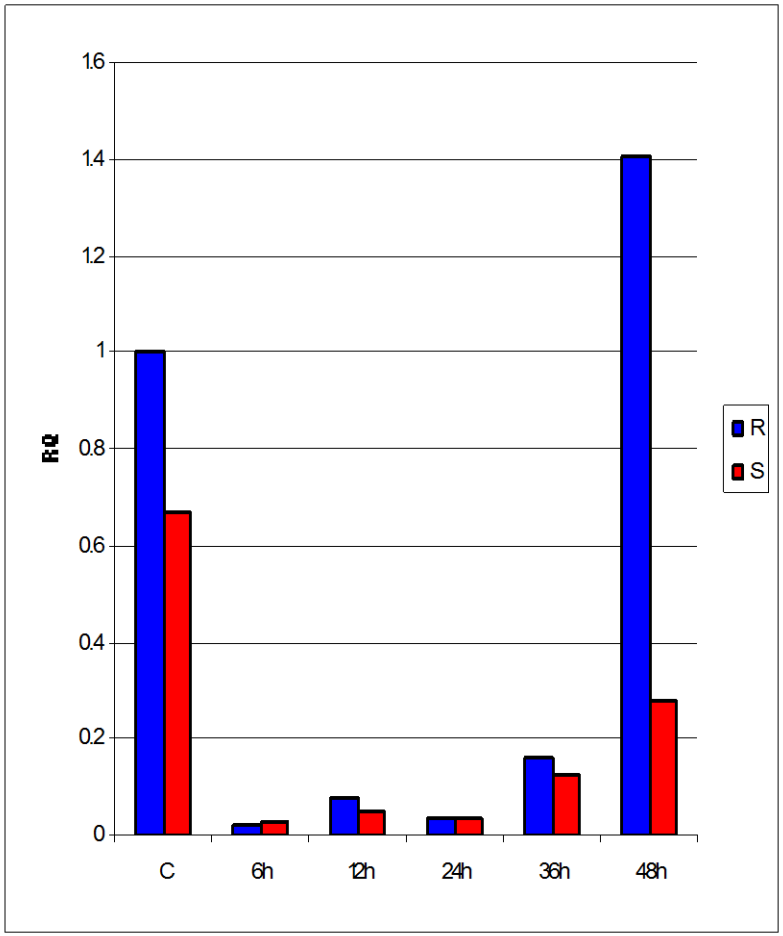
Functional groups of transcripts from SSH cDNA library	Percentage of transcripts abundance in each functional group		
	12 h Library	36 h Library	72 h Library
Signal Transduction	37	17	11
Defense	14	42	15
Cell metabolism	19	24	22
Transcription factors	2	2	11
Transport	5	-	4
Nucleotide metabolism	13	5	11
Cell structure & rigidity	2	6	4
Protein-protein interaction	5	3	9
Fatty acid metabolism	3	-	-
Carbohydrate metabolism	-	-	7
Unknown	-	1	6

Validation of chitinase, TLP and PR5 in Co 93009 and CoC 671 by qRT-PCR

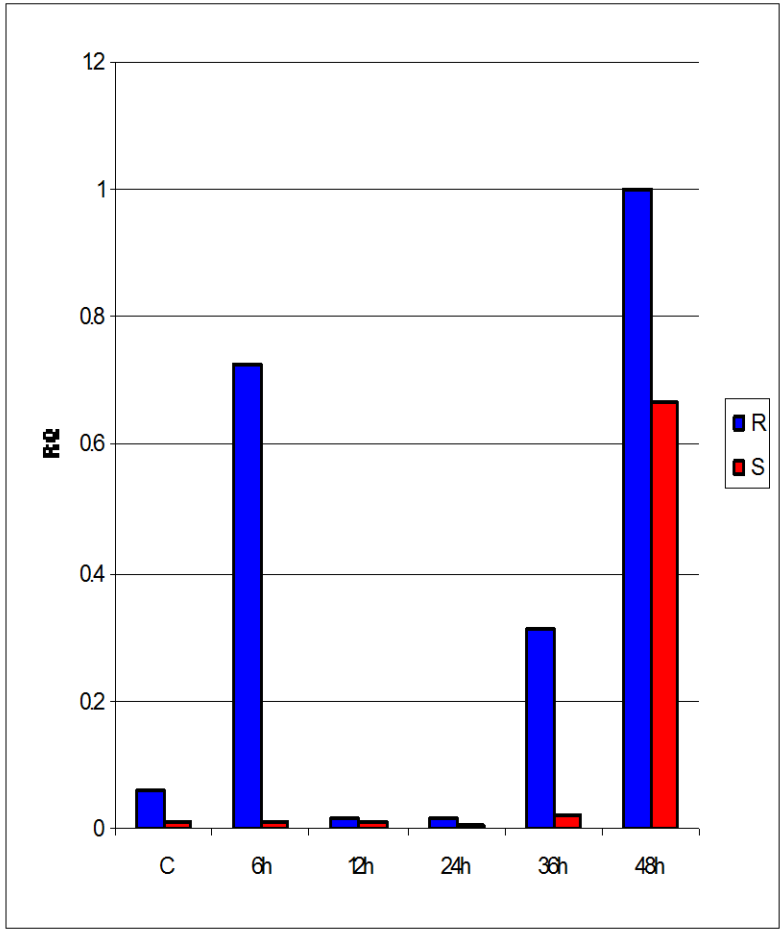


Relative quantitation of differentially expressed transcripts by qRT-PCR

Oxoglutarate/malate translocator



F box protein



R – Resistant (Co 93009)

S – Susceptible (Co*C* 671)

Identification of *C. falcatum* genes in sugarcane matched with plant genes of known functions

Clone name	BLAST sequence similarity	Accession no	Induction in library	E-value
Defence related transcripts				
C-2	Pathogenesis related protein 5 (*Zea mays*)	TA 26133_4547	4 intervals pooled	1.4E-43
LHC-371	Chitinase 3 (*Triticum aestivum*)	DV799658	72h	4.50E-008
C-108	F-box family protein (*Oryza sativa*)	TA45884_4547	4 intervals pooled	1.20E-040
36C-30	Pathogenesis related protein 10 (*Zea mays*)	TA25657_4547	36h	1.40E-053
36C-40	Dirigent like protein (*Saccharum* hybrid cultivar)	TA966_128810	36h	5.10E-056
36C-117	Superoxide dismutase (Mn) mitochondrial precursor (*Zea mays*)	CA185179	36h	2.50E-034
12C-104	Putative peptide methionine sulfoxide reductase	CA223118	12h	2.10E-043
36C-5	Serine protease inhibitor (*Oryza sativa*)	TA32966_4547	36h	1.20E-113
36C-7	Glutathione transferase (*Zea may*)	TA27896_4547	36h	2.00E-047
36C-172	Beta 2 subunit of 20S proteasome (*Oryza sativa*)	CA150453	36h	1.10E-048
12C-2	Putative FH protein interacting protein FIP2 (*Oryza sativa*)	BU572360	12h	1.20E-018
LHC-299	Adenosine kinase like protein (*Triticum aestivum*)	CK156624	72h	1.80E-007

Cell and energy metabolism				
12C-34	ATP synthase beta subunit/transcription terminatin factor rho-like (*Oryza sativa*)	TA42951_4547	12h	1.40E-041
C-5	Mitochondrial 2-oxoglutarate/malate translocator (*Saccharum officinarum*)	AY644469.1	4 intervals pooled	2.00E-094
C-104	S-adenosyl methionine decarboxylase (*Saccharum officinarum*)	GQ380443.1	4 intervals pooled	6.00E-036
C-152	Hemoglobin (*Zea mays*)	BT040115.1	4 intervals pooled	2.00E-052
LHC-359	Proton translocating pyrophosphatase (*Oryza sativa*)	DR731864	72h	2.50E-006
12C-143	NADH-ubiquinone oxidoreductase subunit 8 (*Reclinomonas americana*)	CA069178	12h	6.70E-023
36C-8	Ca2+ATPase (*Oryza sativa*)	TA36207_4547	36h	4.30E-046
36C-22	Cysteine proteinase 1 precursor (*Zea mays*)	CA235310	36h	5.70E-075
36C-29	Putative aconitate hydratase (*Oryza sativa*)	TA25570_4547	36h	4.90E-050
36C-33	Cytidylyltransferase (*Medicago truncatula*)	CA281405	36h	3.60E-045
36C-166	Putative major facilitator superfamily antiporter (*Oryza sativa)*	TA29462_4547	36h	2.90E-044
Cell wall organization				
36C-78	LIM transcription factor homolog (*Zea mays*)	CA157345	36h	6.10E-017
36C-36	Glycerophosphoryl diester phosphodiesterase like protein (*Oryza sativa*)	CA153093	36h	4.80E-062

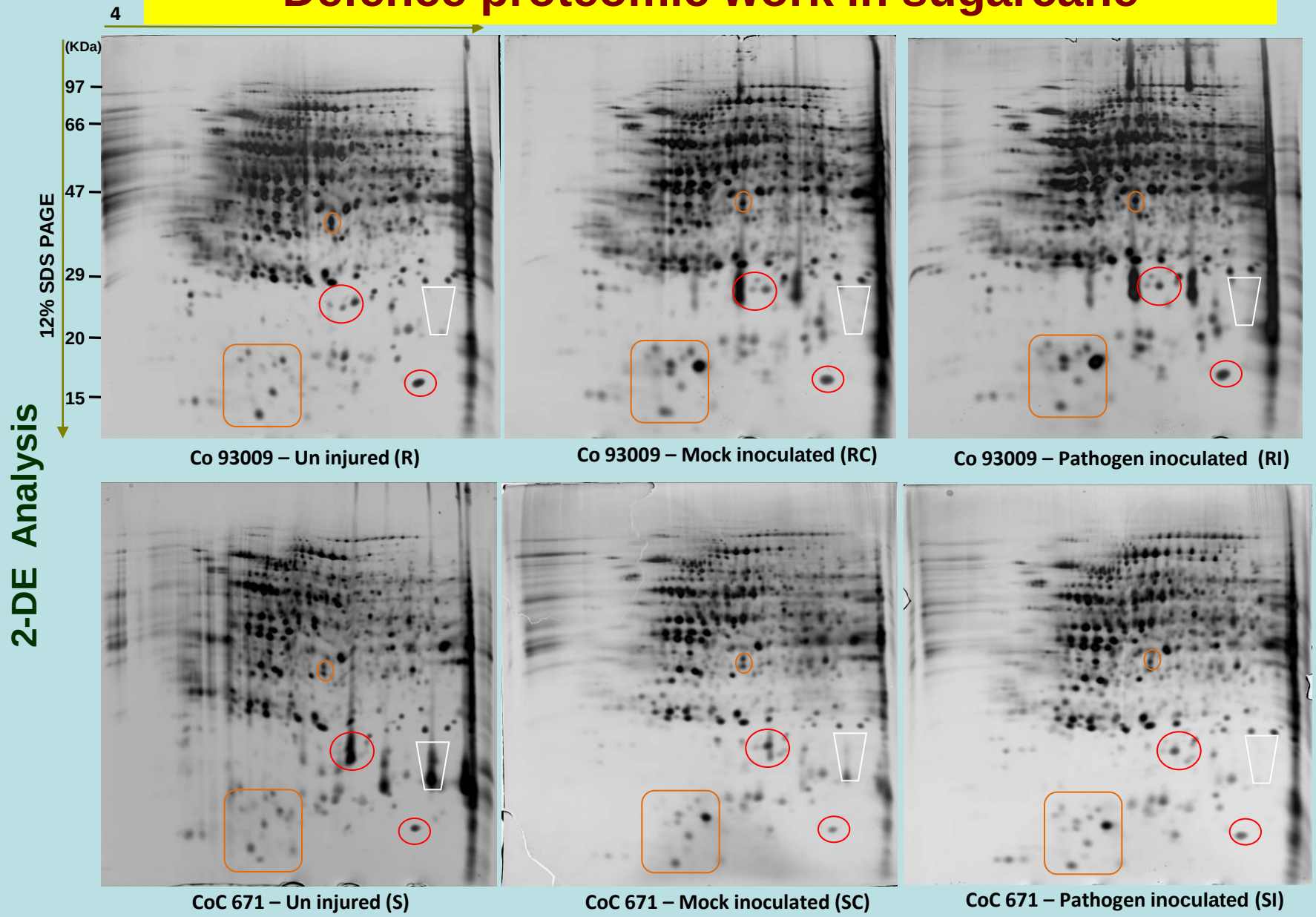
Signal transduction				
12C-53	Calmodulin like protein (*Branchiostoma lanceolatum*)	CA206280	12h	1.70E-037
36C-46	Probable lipoxygenase 4 (*Oryza sativa*)	TA30032_4547	36h	5.20E-030
36C-121	Mitogen activated protein kinase 4 (*Oryza sativa*)	TA29532_4547	36h	1.40E-026
12C-91	Putative ethylene induced calmodulin binding protein 4 (*Oryza sativa*)	TA1407_128810	12h	2.20E-018
36C-128	Farnesylated protein 1 (*Hordeum vulgare*)	TA23444_4547	36h	6.00E-032
Protein folding				
36C-1	Putative peptidyl prolyl cis-trans isomerase NIMA interacting 4 (*Oryza sativa*)	TA35077_4547	36h	8.30E-084
36C-68	Adenylate kinase B (*Oryza sativa*)	TA23016_4558	36h	6.30E-014
36C-48	Putative WD repeat protein (*Oryza sativa*)	TA29322_4558	36h	2.10E-025
12C-73	Helix loop helix like protein (*Oryza sativa*)	TA33525_4547	12h	1.40E-049
36C-21	GRF zinc finger family protein (*Oryza sativa*)	CA115867	36h	1.70E-025
Transport				
12C-64	Importin beta 2 (*Oryza sativa*)	TA30436_4547	12h	7.00E-013
36C-119	High affinity potassium transporter(Common reed)	CA245101	36h	3.20E-042
12C-16	acyl-(acyl-carrier-protein) desaturase chloroplast precursor (*Oryza sativa*)	TA22185_4558	12h	5.40E-069

Potential candidate cDNAs associated with red rot resistance

- 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
- Putative hydroxyproline-rich glycoprotein

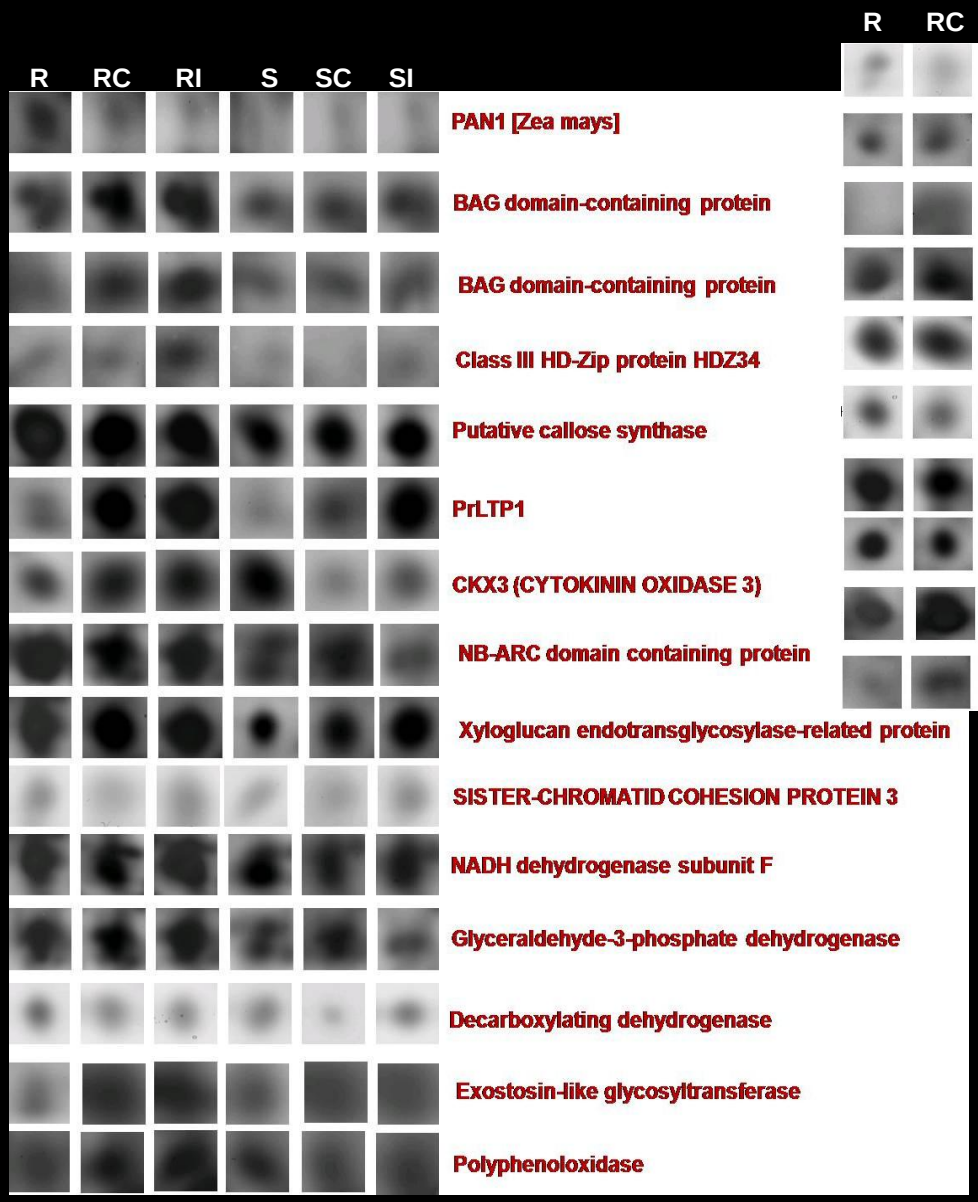
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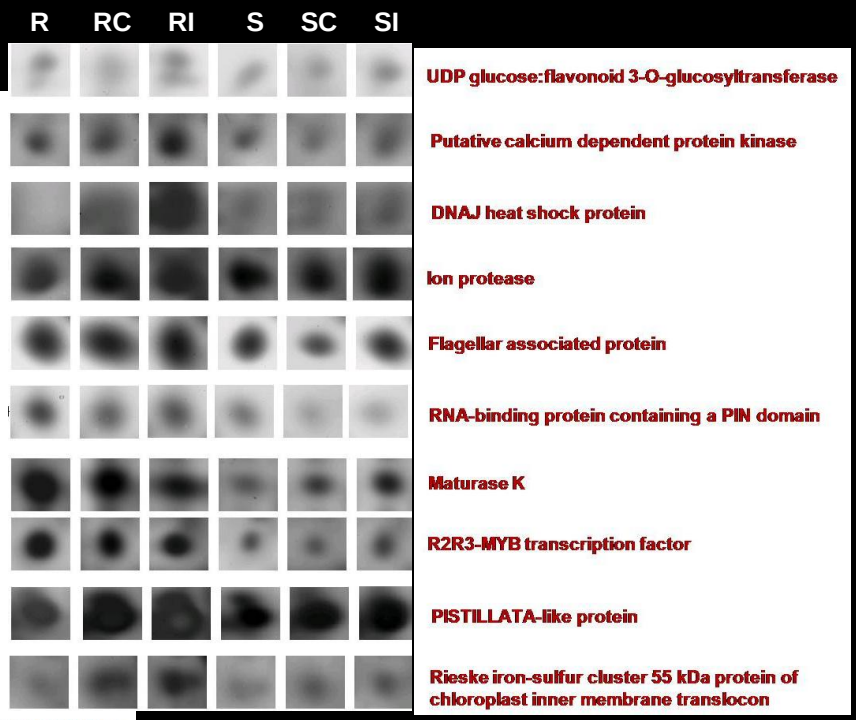
Defence proteomic work in sugarcane



2-DE separation of total soluble proteins from sugarcane stalk tissues (24 after pathogen inoculation). All the gels were separated in the first dimension using pH 4-7 IPG strips and 12% SDS-PAGE in the second dimension, stained with silver staining. Boxes inside the gel are highlighting some of the differential spots.

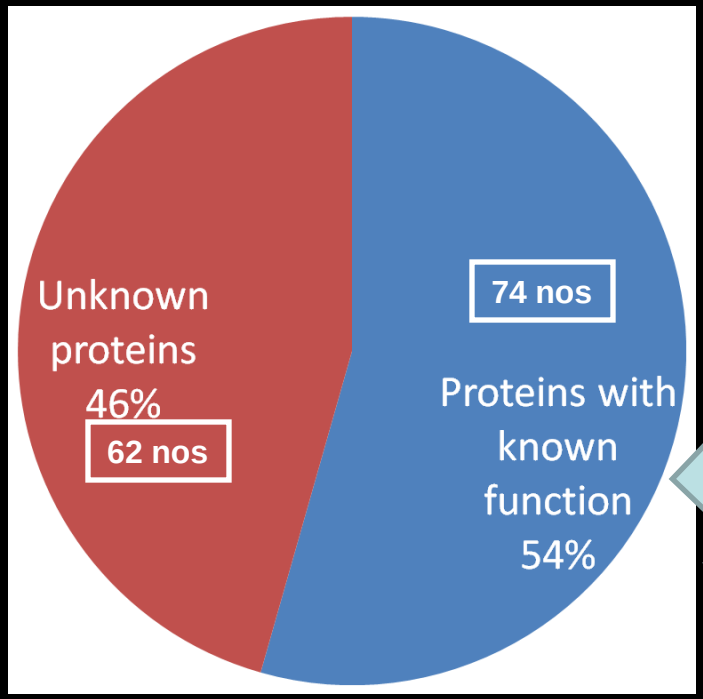
Selected protein spots identified by MALDI-TOF

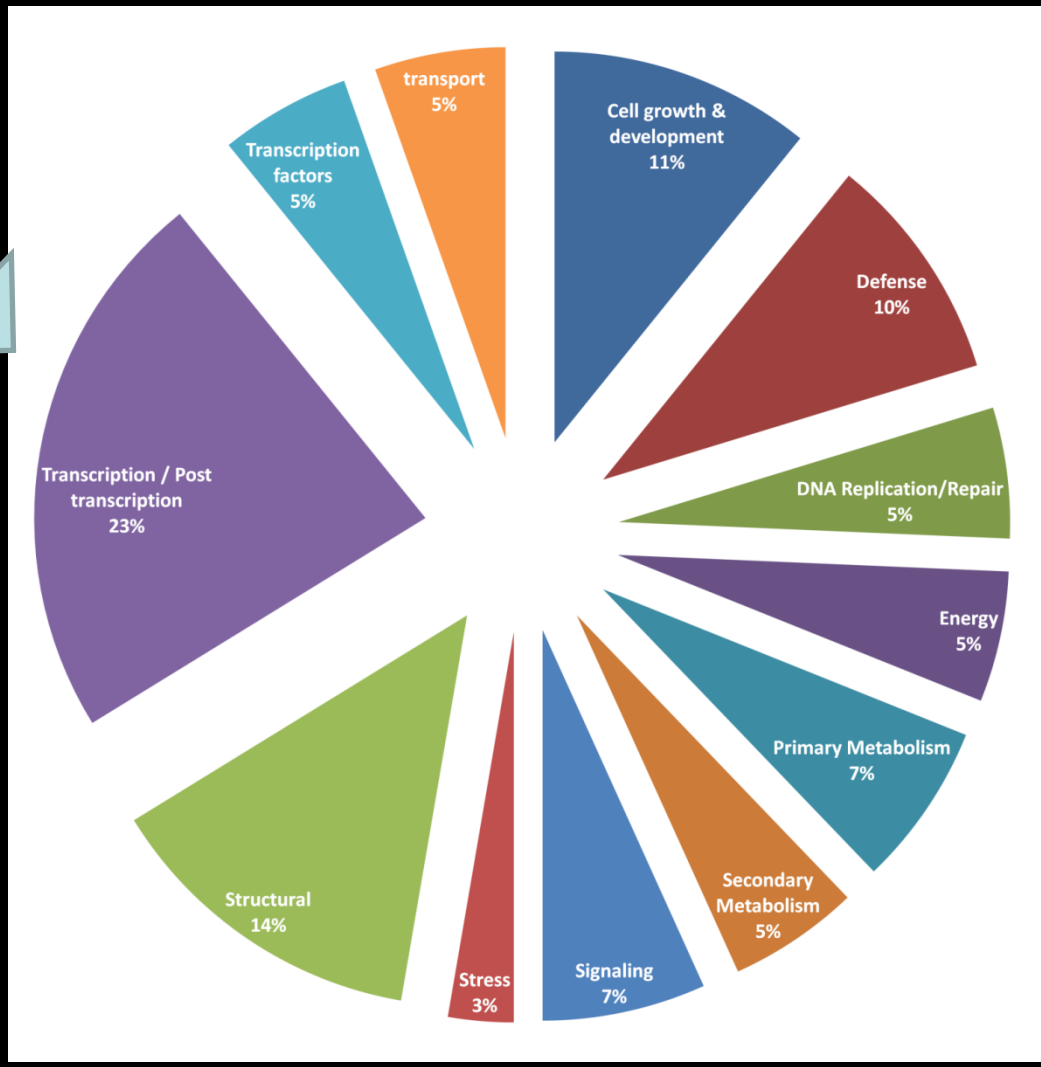




R = Resistant Healthy Control
RC = Resistant mock inoculated control
RI = Resistant Red rot spore inoculated
S = Susceptible Healthy Control
SC = Susceptible mock inoculated control
SI = Susceptible Red rot spore inoculated

Classification of MALDI identified protein spots

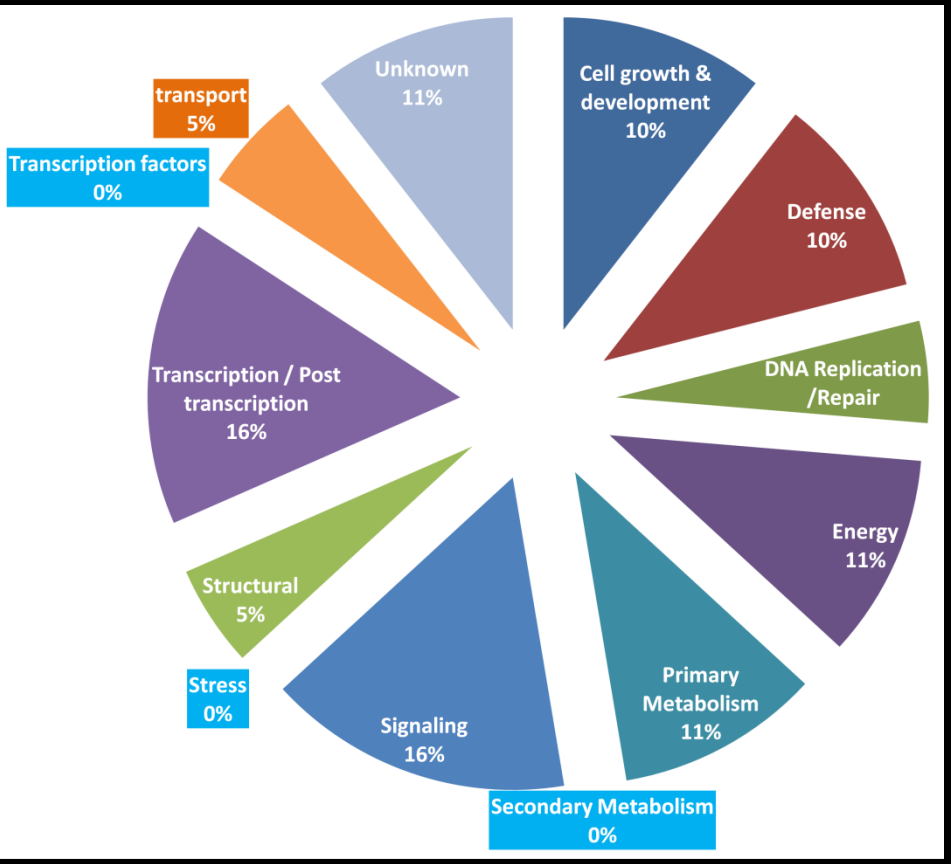




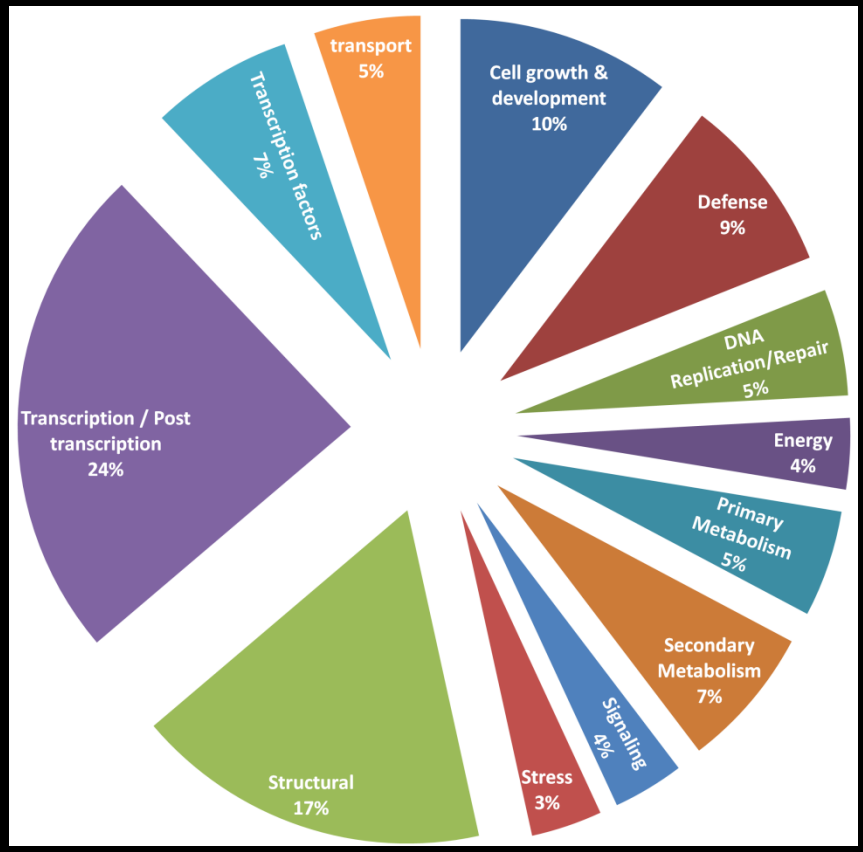
- Large number of proteins belongs to unknown group
- Unknown proteins have match with database proteins but their function is unknown
- Characterization of specific function of proteins will substantially improve the proteomic studies.

Classification of MALDI identified protein spots – based on expression

Down regulated proteins

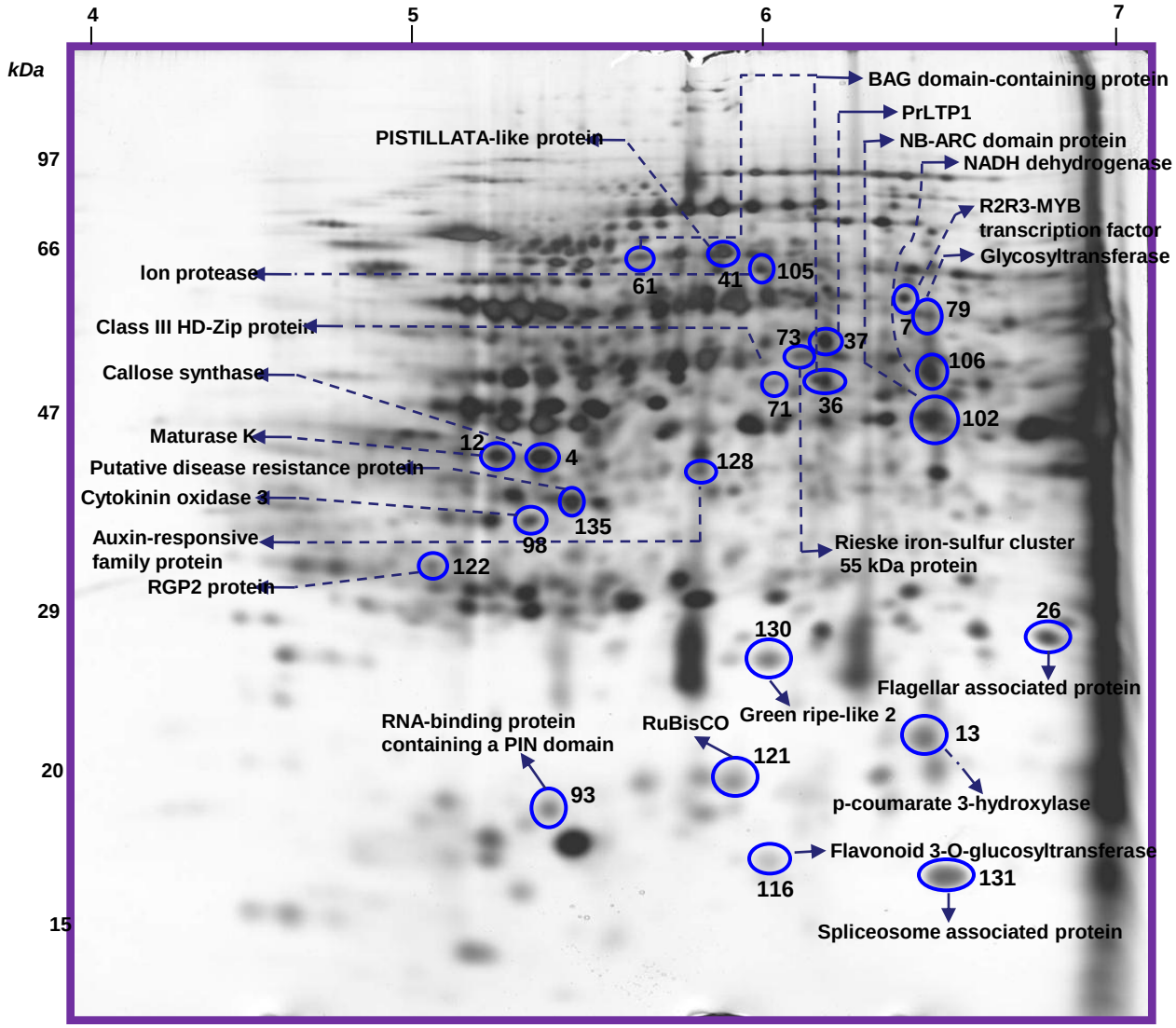


Up-regulated proteins



- Transcripts belonging to transcription factor, secondary metabolism and stress are completely missing from the down-regulated list
- Transcripts belonging to transcription/Post transcription are the major category induced by pathogen inoculation.

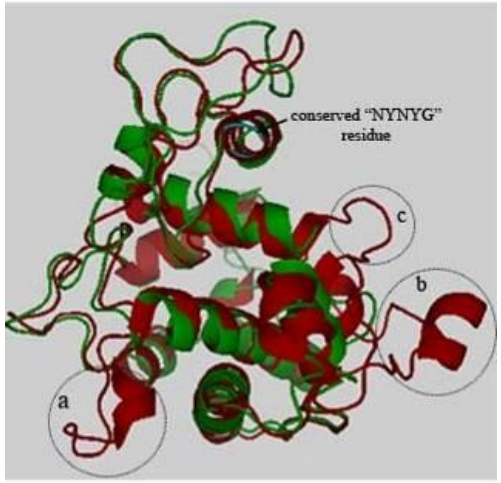
The 2-D gel reference map of sugarcane stalk tissue challenged with *C. falcatum*



High-quality protein spots successfully assigned by tandem MS/MS and database searches were only circled. Protein assignment of identified spots are also mentioned.

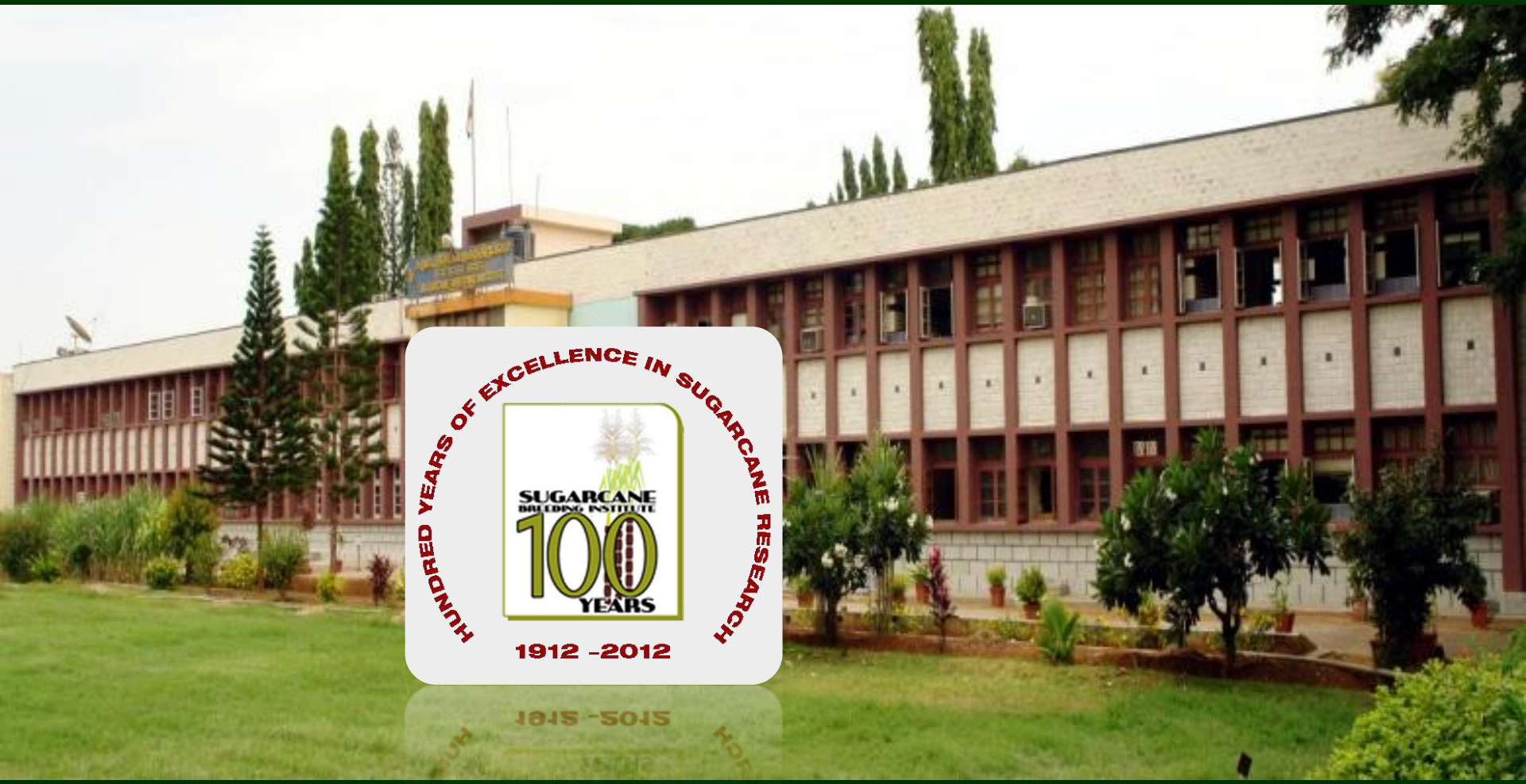
Summary and Conclusion

- Genomic and proteomic tools have improved our understanding on red rot resistance mechanism in sugarcane.
- Many transcripts and proteins involved in red rot resistance have been identified
- Full length of few candidate transcripts have been identified through RACE
- Validation of the differentially expressed transcripts through qRT-PCR is being continued to identify the candidate transcripts
- Studies are in progress to functionally validate the candidate transcripts in red rot resistance in sugarcane by VIGS



3-D structure of sugarcane chitinase superimposed with rice class I chitinase (2DKV) as template

Acknowledgements


HUNDRED YEARS OF EXCELLENCE IN SUGARCANE RESEARCH
SUGARCANE BREEDING INSTITUTE
100 YEARS
1912 -2012

THANK YOU

RV