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# Transient expression of βC1 protein differentially regulates host genes related to stress response, chloroplast and mitochondrial functions

Saiqa Andleeb, Imran Amin, Aftab Bashir, Rob W Briddon, Shahid Mansoor*

## Abstract

**Background:** Geminiviruses are emerging plant pathogens that infect a wide variety of crops including cotton, cassava, vegetables, ornamental plants and cereals. The geminivirus disease complex consists of monopartite begomoviruses that require betasatellites for the expression of disease symptoms. These complexes are widespread throughout the Old World and cause economically important diseases on several crops. A single protein encoded by betasatellites, termed βC1, is a suppressor of gene silencing, inducer of disease symptoms and is possibly involved in virus movement. Studies of the interaction of βC1 with hosts can provide useful insight into virus-host interactions and aid in the development of novel control strategies. We have used the differential display technique to isolate host genes which are differentially regulated upon transient expression of the βC1 protein of chili leaf curl betasatellite (ChLCB) in *Nicotiana tabacum*.

**Results:** Through differential display analysis, eight genes were isolated from *Nicotiana tabacum*, at two and four days after infiltration with βC1 of ChLCB, expressed under the control of the *Cauliflower mosaic virus* 35S promoter. Cloning and sequence analysis of differentially amplified products suggested that these genes were involved in ATP synthesis, and acted as electron carriers for respiration and photosynthesis processes. These differentially expressed genes (DEGs) play an important role in plant growth and development, cell protection, defence processes, replication mechanisms and detoxification responses. Kegg orthology based annotation system analysis of these DEGs demonstrated that one of the genes, coding for polynucleotide nucleotidyl transferase, is involved in purine and pyrimidine metabolic pathways and is an RNA binding protein which is involved in RNA degradation.

**Conclusion:** βC1 differentially regulated genes are mostly involved in chloroplast and mitochondrial functions. βC1 also increases the expression of those genes which are involved in purine and pyrimidine metabolism. This information gives a new insight into the interaction of βC1 with the host and can be used to understand host-virus interactions in follow-up studies.

## Background

Geminiviruses are economically important plant pathogens and are characterized by twinned isometric particles containing single-stranded (ss)DNA genomes of 2.5-3.0 kb [1] that replicate through double-stranded (ds)DNA intermediates by a rolling-circle mechanism [2]. The family *Geminiviridae* is divided into four genera, (*Begomovirus*, *Mastrevirus*, *Curtovirus* and *Topocuvirus*) that encompass viruses that differ in genome organization as well as their insect vectors. Begomoviruses are transmitted by the whitefly *Bemisia tabaci* and have either monopartite or bipartite genomes. Monopartite begomoviruses are often associated with circular, ssDNA satellites that are collectively referred to as betasatellites (formerly known as DNA β). Betasatellites have recently been found to be associated with some bipartite begomoviruses and are required by some of their helper begomoviruses to induce *bona fide* disease symptoms in plants. Numerous economically important diseases and even the earliest recorded plant

* Correspondence: shahidmansoor7@gmail.com
Agricultural Biotechnology Division, National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan

viral disease are now known to be caused by begomovirus/betasatellite complexes [3,4].

Betasatellites are widespread in the Old World, where monopartite begomoviruses are known to occur. Numerous distinct betasatellites, from various economically important hosts and diverse locations, have been cloned and have been found in most cases to contribute significantly to disease symptoms [5]. Analysis of betasatellite sequences reveals a highly conserved organization consisting of an adenine-rich region and a region of sequence highly conserved between all betasatellites (known as the satellite conserved region [SCR]). The SCR contains a potential hairpin structure with the loop sequence TAA/GTATTAC that has similarity to the origins of replication of geminiviruses and nanoviruses. Betasatellites encode only a single gene, known as the bC1, located on the complementary-sense strand, is conserved in position and size in all betasatellites [6,7].

Chilli leaf curl betasatellite (ChLCB) is associated with chilli leaf curl disease (ChLCuD), a significant constrain to chilli production across the Indian subcontinent [8,9]. Saeed et al. [5] demonstrated that tobacco plants transformed with the βC1 of Cotton leaf curl Multan betasatellite (CLCuMB) under the control of the *Cauliflower mosaic virus* 35S promoter, or with a dimer of CLCuMB, exhibited severe disease-like phenotypes, while plants transformed with a mutated version of the βC1 appeared normal. Qazi et al. [10] showed that expression of CLCuMB βC1 from a *Potato virus X* vector induced symptoms typical of cotton leaf curl disease (CLCuD) in the absence of the helper begomovirus. These results demonstrated that CLCuMB βC1 is the major determinant of symptoms of the CLCuD complex [10].

The interactions between plants and viruses are complex and involve several types of responses that may or may not cause disease in the host [11]. In compatible interactions, the invading virus is able to infect and replicate within a susceptible plant to cause disease. Alternatively, the host may trigger innate immunity mechanisms that restrict virus movement and prevent disease onset. In both situations, viral pathogens severely disturb plant growth and development, due to their effect on cellular metabolism [11]. Viral infection produces a plethora of symptoms derived from biochemical and metabolic changes in cells, tissues and even in the whole plants which are susceptible and hypersensitive resistant hosts. Huang et al. [12] and Sui et al. [13] demonstrated that plant viruses cause severe impact on host gene expression and protein activity due to the activation of a set of genes and the inactivation of others. The gene expression profile in the host plant changes according to the timing and localization of the infection, as the virus spreads from cell to cell away from the site of inoculation [14,15].

The present studies are aimed at identifying host genes and pathways that are induced by ChLCB βC1. This may be achieved using differential RNA display technology. This technique is based on "differential display reverse transcriptase polymerase chain reaction" (DDRT-PCR), first described by Liang and Pardee [16]. This method has the advantage of technical simplicity, a lower bias against rare messages and a requirement of only small quantities of starting mRNA. Several modifications of the original technique have been reported with some solutions to the key problems identified by some authors [17]. Stress responses have been studied using DDRT-PCR in *C. elegans* and *S. cerevisiae* [18-20]. DDRT-PCR has been applied in many laboratories to identify genes involved in signal cascades.

The identification of host genes affected by ChLCB βC1 may provide useful insights into virus-host interactions and provide targets for novel control strategies. By differential display analysis we have identified *N. tabacum* genes differentially regulated in response to the transient expression of ChLCB βC1 protein. Subsequently the effects of βC1 expression on each gene identified were verified by quantitative real time PCR analysis.

## Results and Discussion

We have made a further modification of the DDRT-PCR technique by utilizing the mRNA fraction instead of total RNA and by resolving the products of DDRT-PCR on 1% agarose gels stained with ethidium bromide [16]. We have identified several genes which were differentially expressed at 2 dpi and 4 dpi. Two different concentrations of cDNA (100 ng/μl and 10 ng/μl; Figures 1A-F) were used, of which ninety seven differentially expressed genes (DEGs) were amplified by different anchored and arbitrary primer pairs (Table 1; Figures 1A-F). The anchored and arbitrary are random decamer primers, and used as reverse and forward primer for cDNA synthesis. Agroinfiltration was used for transient expression of βC1 (ChLCB) under 35S promoter. DDRT-PCR showed different bands of transcripts in comparison to control plants. Some of the primer combinations did not yield an amplification product (Figures 1A-F). At 2 dpi no difference was observed in control and infected plants as indicated in DD10 (B7, B18); DD11 (B15, B16, B19); and DD12 (B11, B19), respectively (Figures 1A-C). On the other hand, at 4 dpi same pattern was also observed in DD10 (B2, B3, B4, B8, B10, B14, B17, B18, B20), DD11 (B2, B6, B7, B9, B10, B11, B12, B13, B16, B17, B18), and DD12 (B6, B7, B11, B14, B15) respectively (Figures 1D-F).

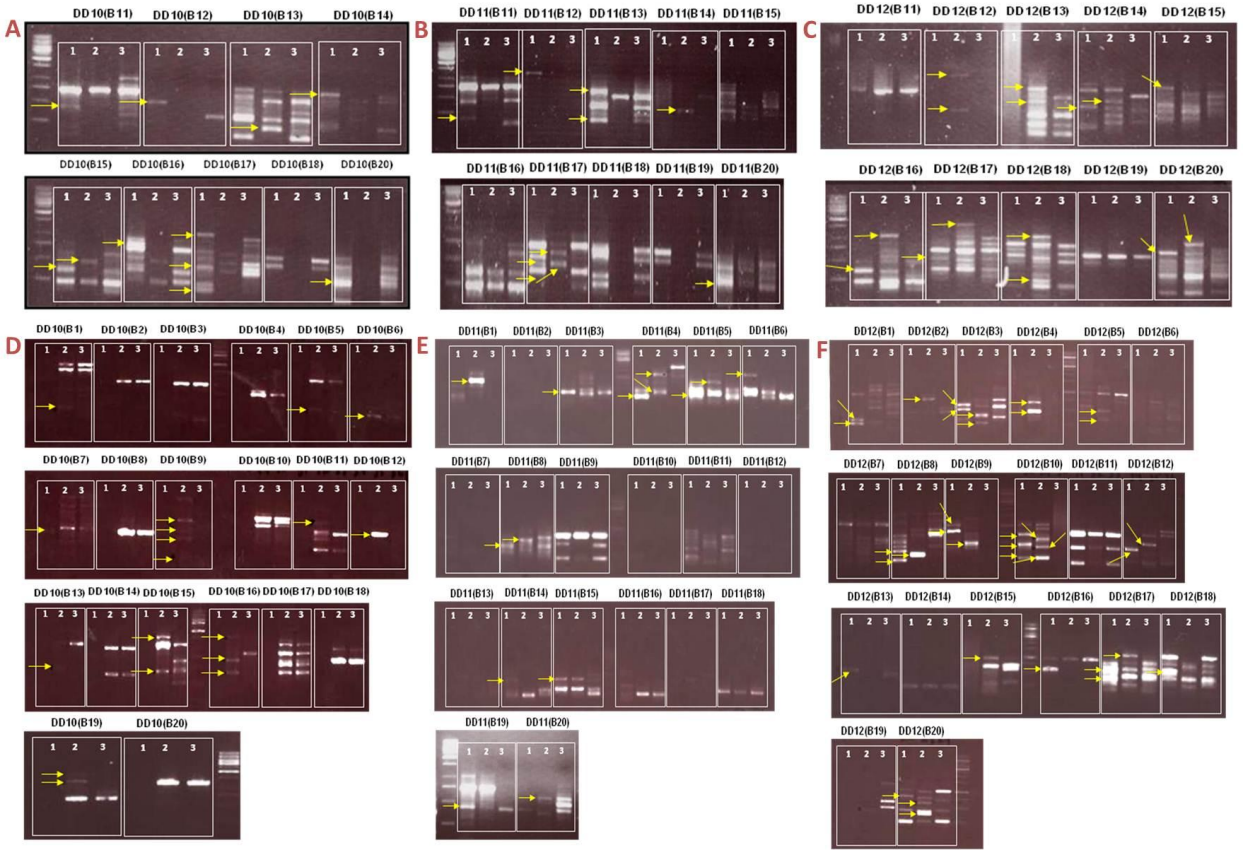


**Figure 1 A. Identification of genes differentially expressed in response to βC1 by differential display analysis at two and four days after infiltration**. In each combination of arbitrary and anchored primers, lane 1 represents 100 ng/μl of cDNA (pSAβC1pGreen0029), lane 2 shows 10 ng/μl of cDNA (pSAβC1pGreen0029) and lane 3 indicates 100 ng/μl pGreen0029 in *Agrobacterium tumefaciens* strain GV 3101. Differential display analysis two days after inoculation with DD10 (B11-B20); B. DD analysis two days after inoculation with DD11 (B11-B20); C. DD analysis after two days of inoculation with DD12 (B11-B20); D. DD analysis after four days of inoculation with DD10 (B1-B20); E. DD analysis four days after inoculation with DD11 (B1-B20); F. DD analysis four days after inoculation with DD12 (B1-B20). The bands eluted for analysis are indicated (→).

### Analysis of DEGs identified at two days post infiltration

Differentially expressed products were cloned and sequenced. The identity of these differentially expressed genes was analysed using NCBI nucleotide data blast system. The ratio of differentially expressed genes (SA1, SA2, SA3, SA4, SAA, SAB, SAC and SAD) expressed in a sample versus a calibrator (healthy plant and plant infiltrated with pGreen0029) in comparison to a reference gene (rubisco) is indicated in the Tables 2, 3 and 4. The results of Delta Delta (Ct), Livak and the Pfaffi mathematical models indicated that SAA, SAB, SAD, SA1, SA2, and SA3 mRNA expression were upregulated in sample compared to the calibrator (plant inoculated with pGreen0029 and healthy plant). Interestingly elevation of mRNA transcripts was also detected by RT-PCR (Figure 2A and 2B). In contrast SAC and SA4 mRNA expression was down regulated in the sample compared to the calibrator (Figure 2A and 2B). The calculated expression levels by these models is indicated in the Tables 2, 3 and 4.

The results indicated that SAA showed 76% nucleotide sequence identity with *Solanum lycopersicum* WRKY transcription factor IId-1 splice. The results show that the SAA gene is upregulated (Figure 2) upon inoculation with the ChLCuB βC1 gene, which is a pathogenicity determinant [21-23], helps in viral movement, is involved in symptom induction [9,24,25], is a suppressor of gene silencing [26] and may be the target of a host response that up-regulates WRKY transcription factors [27]. It has been shown that the transcription of WRKY genes are strongly and rapidly upregulated in response to wounding, pathogen infection or abiotic stresses in numerous plant species, as indicated in Figure 3[28]. Infection of tobacco with *Tobacco mosaic virus* (TMV) or bacteria, or treatment with fungal elicitors, salicylic acid (SA) or $H_2O_2$, strongly induces several WRKY genes [29,30].

**Table 1 Sequences of oligonucleotide primers used in the study**

| Use | Primer | Sequence |
|---|---|---|
| | Anchored Primer | |
| | DD10 | 5'-TTTTTTTTTTG-3' |
| cDNA | DD11 | 5'-TTTTTTTTTTC-3' |
| | DD12 | 5'-TTTTTTTTTTA-3' |
| | Arbitrary Primer | |
| | B-01 | 5'-GTTTCGCTCC-3' |
| | B-02 | 5'-TGATCCCTGG-3' |
| | B-03 | 5'-CATCCCCCTG-3' |
| | B-04 | 5'-GGACTGGAGT-3' |
| | B-05 | 5'-TGCGCCCTTC-3' |
| | B-06 | 5'-TGCTCTGCCC-3' |
| | B-07 | 5'-GGTGACGCAG-3' |
| | B-08 | 5'-GTCCACACGG-3' |
| | B-09 | 5'-TGGGGGACTC-3' |
| DDRT-PCR | B-10 | 5'-CTGCTGGGAC-3' |
| | B-11 | 5'-GTAGACCCGT-3' |
| | B-12 | 5'-CCTTGACGCA-3' |
| | B-13 | 5'-TTCCCCCGCT-3' |
| | B-14 | 5'-TCCGCTCTGG-3' |
| | B-15 | 5'-GGAGGGTGTT-3' |
| | B-16 | 5'-TTTGCCCGGA-3' |
| | B-17 | 5'-AGGGAACGAG-3' |
| | B-18 | 5'-CCACAGCAGT-3' |
| | B-19 | 5'-ACCCCCGAAG-3' |
| | B-20 | 5'-GGACCCTTAC-3' |

This suggests that the expression of βC1 gene results in a stress response and the plant responds to these stresses by increasing the transcription of WRKY genes.

SAB showed 68% nucleotide sequence identity with *A. thaliana* putative Rieske iron-sulfur protein (RISP) and 73% with *P. sativum* RISP. The expression of SAB was upregulated in the response of ChLCB βC1 (Figure 2). RISP was identified from expression of betaC1 gene [31] and is absolutely required for mitochondrial respiration (Figure 3) as reported earlier [32,33]. Mitochondrial RISP is encoded by a nuclear gene, translated as a precursor protein in the cytoplasm and post-translationally imported into mitochondria. Huang [34] demonstrated that the RISP gene family is differentially regulated; higher RISP levels occur in flowers than in leaves, stems and roots. RISP is involved in energy production in the form of ATP, required for pollen development and must be supplied mainly by mitochondria. Similarly, flower mitochondria could meet the high demand for energy either by increasing their metabolic activity to generate more ATP per mitochondrion or by increasing their number per cell so that more ATP is produced [35]. It has been shown that expression of βC1 results in foliar enations [10], which is an indication of enhanced cell division. Cell division is an energy requiring process. Therefore one possible pathway to acquire energy is via RISP proteins (Figure 3). However, this hypothesis will require further experimental confirmation.

The SAC DNA sequence shows 93% nucleotide sequence identity with both NADH dehydrogenase subunit 1 (*ndh*1) and NADH dehydrogenase subunit 2 (*ndh*2) of *N. tabacum* mitochondrial genes, also known as NADH oxidoreductase. Similar to the SAB, it has been demonstrated that SAC is a *N. tabacum* mitochondrial protein and also involved in generation of cellular energy in the form of ATP by building the electrochemical potential in electron transport chain as indicated in (Figure 3) [35,36]. The SAD transcript showed 47% nucleotide sequence identity with the *M. truncatula* quinon protein alcohol dehydrogenase. The quinon protein alcohol dehydrogenases are involved in plant development and senescence, reducing the concentration of toxic amines during stress conditions, and providing hydrogen peroxide for wall stiffening and lignification (Figure 3).

**Table 2 Conclusion of relative quantification methods of differentially expressed genes at two and four days after inoculation**

| DEG | Length bps | Identity | Up/Down regulation |
|---|---|---|---|
| **SAA** | (287) | *S. lycopersicum* WRKY transcription factor IId-1 splice | Upregulated |
| **SAB** | (231) | Putative Rieske iron-sulfur protein [*A. thaliana*]Length = 539, Rieske iron-sulfur protein Tic55 [*P. sativum*]Length = 553 | Upregulated |
| **SAC** | (386) | *N. tabacum* mitochondrial DNA, complete genome Length = 430597 NADH dehydrogenase subunit 1 NADH dehydrogenase subunit 2, 846 bp at 5' side: | Down regulated |
| **SAD** | (262) | Quinonprotein alcohol dehydrogenase like *M. truncatula* | Upregulated |
| **SA1** | (442) | Trigger factor (chaperone in protein export) | Upregulated |
| **SA2** | (688) | *A. thaliana* calmodulin-binding receptor-like kinase | Upregulated |
| **SA3** | (772) | Polyribonucleotide nucleotidyltransferase | Upregulated |
| **SA4** | (283) | Chromosomal replication initiator protein DnaA | Down regulated |

**Table 3 Relative quantification methods of differentially expressed genes two days post inoculation**

| Genes | Identity | Relative quantification against Unit mass | | Relative quantification Normalized to a reference gene | | |
|---|---|---|---|---|---|---|
| | | Control | Healthy | Livak method | ΔCT Method | Pfaffi Method |
| **SAA** | *S. lycopersicum* WRKY transcription factor IId-1 splice | 2.32 | 1.32 | 0.737C/ 0.381H | 0.942C/ 0.838H | 0.737C/ 0.381H |
| **SAB** | Putative Rieske iron-sulfur protein [*A. thaliana*]Length = 539, Rieske iron-sulfur protein Tic55 [*P. sativum*]Length = 553 | 2.751 | 0.566 | 0.870C/ 0.162H | 0.972C/ 0.728H | 0.870C/ 0.162H |
| **SAC** | *N. tabacum* mitochondrial DNA, complete genome Length = 430597 NADH dehydrogenase subunit 1 NADH dehydrogenase subunit 2, 846 bp at 5' side | 0.010 | 1.905 | 0.003C/ 0.547H | -0.672C/ 1.355H | 0.003C/ 0.547H |
| **SAD** | Quinonprotein alcohol dehydrogenase like [*M. truncatula*] | 5.205 | 0.829 | 1.647C/ 0.238H | 1.167C/ 0.707H | 1.647C/ 0.238H |

### Analysis of DEGs identified at four days post infiltration

Several genes were also identified that were differentially expressed at 4 dpi. DEG SA1 shows 99% nucleotide sequence identity with trigger factor (chaperone in protein export) of *P. acnes*. It has been suggested that molecular chaperones play a critical role in targeting proteins to the mitochondria, are involved in $Ca^{+}$ dependent signaling pathway (Figure 3) and in the subsequent folding of the imported protein [37-39]. It may be very useful to analyze the interaction of βC1 with chaperones through protein-protein interaction in future. It has been shown that SA2 transcript belongs to the primary calcium receptor called calmodulin (CaM; Figure 3), which is a ubiquitous protein found in both plants and animals [40]. It is located in cytoplasmic and nuclear compartments and can be attached to the plasma membrane in plant cells [41,42].

SA2 showed 72% nucleotide sequence identity with *A. thaliana* calmodulin-binding receptor-like kinase 2 (CRCK2) and, interestingly, the expression of CRCK1 is up-regulated by cold and salt stresses, as well as the stress molecules ABA (abscisic acid) and hydrogen peroxide, suggesting that CRCK2 may be involved in osmotic and oxidative stress signal transduction pathways in plants [43]. It has been suggested that CRCK2 protein is up regulated (Figure 2) during pathogen infection and also regulates the activities of a wide range of CaM binding proteins (CaMBPs), including metabolic enzymes, transcription factors such as WRKY group II d [44], ion channels, protein kinases/phosphatases and structural proteins [45,46], as indicated in Figure 3.

Transcript SA3 showed 92% nucleotide sequence identity with the polynucleotide nucleotidyltransferase from *P. cryohalolentis K5* (PNPase; encoded by the pnp gene). PNPase is an RNA binding protein, involved in post-transcriptional gene silencing, participates in RNA degradation [47] and plays a central role in adaptation to growth at low temperature [48]. Previous studies identified PNPase in eubacteria [49-51], *Drosophila melanogaster* [52], plants [53,54], and even mice and humans [55,56]. Here it has been identified in *N. tabacum* in the response of βC1 of ChLCB. SA4 shows 90% nucleotide sequence identity with the chromosomal replication initiator protein DnaA. βC1 induces cell proliferation (enations) and a requirement for DnaA during cell division is thus consistent with this finding. Sequence analysis of the cloned DEGs showed 8 of them to represent genes that have been previously characterized (Table 5), while the remainder represent genes of unknown function and hypothetical proteins predicted from sequence. All these genes are associated with chloroplast and mitochondrial host compartments.

**Table 4 Relative quantification methods of differentially expressed genes four days post inoculation**

| Gene | Identity | Relative quantification against Unit mass | | Relative quantification normalized to a reference gene | | |
|---|---|---|---|---|---|---|
| | | Control | Healthy | Livak method | ΔCT Method | Pfaffi Method |
| **SA1** | Trigger factor (chaperone in protein export) | 2.88 | 17.75 | 1.443C/7.727H | 1.08C/1.74H | 1.443C/7.727H |
| **SA2** | *A. thaliana* calmodulin-binding receptor-like kinase | 3.759 | 3.759 | 8.564 | - | 8.564 |
| **SA3** | Polyribonucleotide nucleotidyltransferase | 1.32 | 1.70 | 0.664C/0.742H | 0.790C/.837H | 0.664C/0.742H |
| **SA4** | Chromosomal replication initiator protein DnaA | 0.659 | 0.882 | 0.329C/0.253H | 0.674C/0.706H | 0.329C/0.253H |

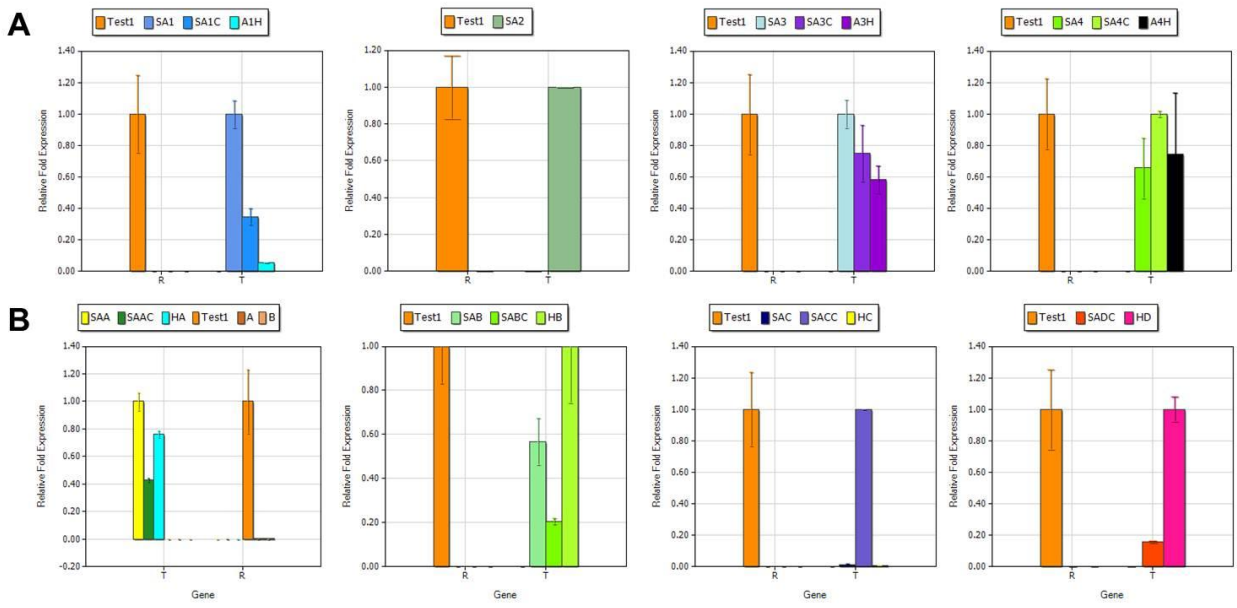


**Figure 2 Quantitative real time RT-PCR analysis of DEGs identified in response to βC1 at two and four days after infiltration of *N. tabacum***. SA1, SA2, SA3, SA4, SAA, SAB, SAC and SAD test samples with color indication represent the up and down regulation of differentially expressed genes as compared to calibrator having only pGreen0029 vector (SA1C, SA3C, SA4C, SAAC, SABC, SACC, SADC) and another healthy calibrator (A1H, A3H, A4H, HA, HB, HC and HD). In both (A) and (B) R stand for reference gene and T for test samples.

The results suggest that the DEGs identified in response to βC1 are involved in multiple pathways; oxidative stress signaling, Ca+ dependent signaling, salicylic acid signaling pathways (Figure 3). Interestingly, these DEGs are related to specific cellular compartments, mitochondria and chloroplasts (Figure 3), where they act as electron carrier for respiration and photosynthesis by ATP synthesis (Figure 3). Collectively these genes perform their roles in plant growth and development, detoxification responses, cell protection and defense against invading viral proteins or pathogen (Figure 3).

### Analysis of DEGs using KOBAS

The DEGs responsive to ChLCB βC1 were analyzed using the KEGG orthology (KO) system, also called KOBAS (KO Based Annotation System). This showed that polyribonucleotide nucleotidyltransferase is involved in the purine and pyrimidine metabolic pathways (Table 6 and 7). These finding suggest that βC1 interact with host genes in such a manner to increase the amount of purines and pyrimidines in the cells and this is required for cell division which is induced by βC1.

## Conclusions

From all these related results it has been concluded that the DEGs in the response of βC1 of ChLCB under 35S cauliflower promoter are related to the chloroplast and mitochondria and are involved in the ATP synthesis, act as electron carriers for respiration and photosynthesis processes. These DEGs play an important role in plant growth and development, cell protection, defence processes, replication mechanisms and detoxification responses as illustrated in Figure 3.

## Methods

### Cloning of βC1 of Chilli leaf curl betasatellite in pJIT163

The βC1 of *ChLCB* was cloned under the control of the cauliflower mosaic virus 35S promoter in the pJIT163 plant expression vector. A set of primers (ChβC135S(F) 5'-GCAAGCTTATGCACCACGTATATGAATTATGTC C-3'/ChβC135S(R) 5'- GCGAATTCTCACACACACA-CATTCGTACATAC-3'; having *Eco*RI and *Hin*dIII restriction sites, respectively) were designed to the reported sequence (accession no. AJ316032) to amplify a 450 bp DNA fragment containing the ChLCB βC1 gene. The fragment was amplified with an initial 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min. A final extension at 72°C for 10 min was included. The amplification product was analyzed by 1% agarose gel electrophoresis. The amplified fragment and pJIT163 vector were restricted with *Eco*RI and *Hin*dIII restriction enzymes at 37°C overnight, precipitated with phenol-chloroform and ligated at 16°C overnight. The ligated product was transformed

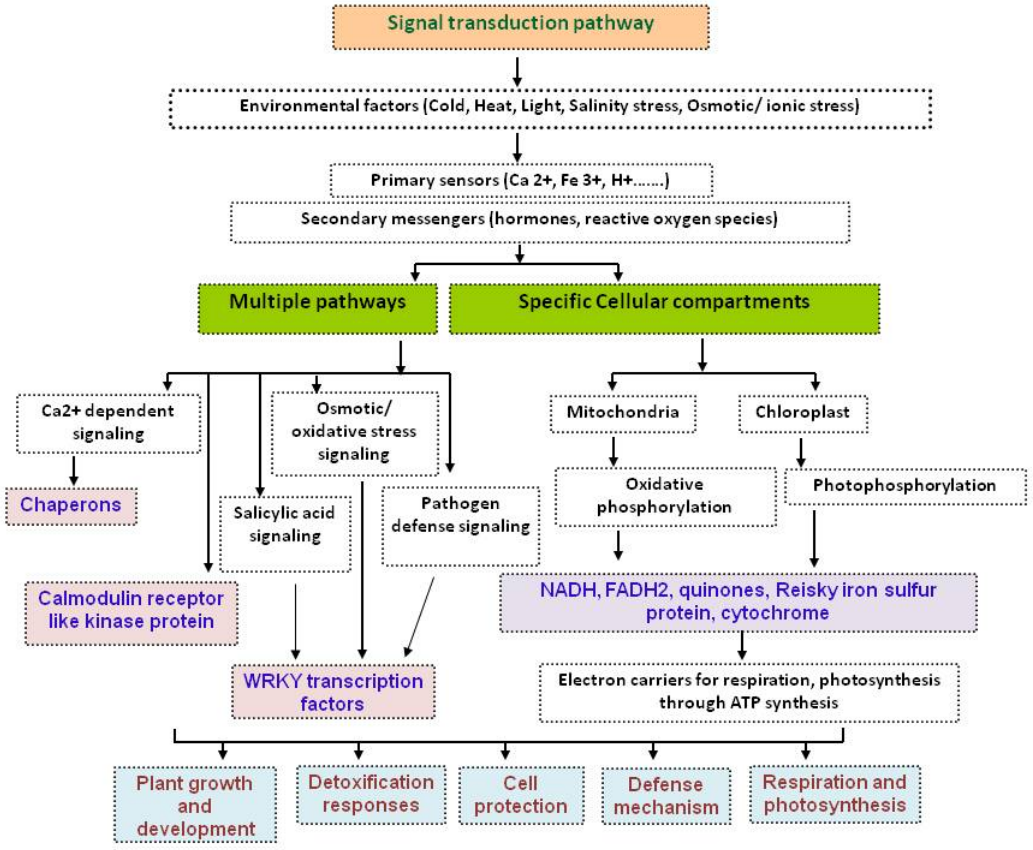


**Figure 3 Schematic pathway showing the involvement of the differentially expressed genes (DEGs) in signal transduction pathways**. DEGs isolated from *N. tabacum* at two and four days after infiltration in response of βC1 are involved in different pathways during host protein interactions and also segregate in specific cellular compartments. Chaperons, CRCK2 and WRKY transcription factors involved in $Ca^{+}$ dependent signalling, salicylic acid signalling, osmotic and oxidative stress signalling and pathogen defence signalling pathways. In contrast, NADH, Reisky iron sulphur protein and quinone protein are related to mitochondrial and chloroplast sysstems and act as electro carriers for respiration, photosynthesis by ATP synthesis. The collective role of these DEGs are in defence, cell protection, respiration, photosynthesis, detoxification, plant growth and development.

**Table 5 Differentially expressed genes (DEGs) and their identities**

| DEG | Length of amplified fragment | Identity | Accession No. | Identity |
|---|---|---|---|---|
| | | **Genes differentially expressed at two days after inoculation** | | |
| **SAA** | 287 | *S. lycopersicum* WRKY transcription factor IId-1 splice | AY157059 | (76%) |
| **SAB** | 231 | Putative Rieske iron-sulfur protein [*A. thaliana*]Length = 539, Rieske iron-sulfur protein Tic55 [*P. sativum*] Length = 553 | NM128041 AJ000520 | (68%)/ (73%) |
| **SAC** | 386 | *N. tabacum* mitochondrial DNA, complete genome Length = 430597: NADH dehydrogenase subunit 1 and NADH dehydrogenase subunit 2, 846 bp at 5' side | BA000042 | (93%) |
| **SAD** | 262 | Quinonprotein alcohol dehydrogenase like [*M. truncatula*] | ABE84009 ABE86610 | (47%) |
| | | **Genes differentially expressed at four days after inoculation** | | |
| **SA1** | 442 | Trigger factor (Chaperone protein) | AE017283 | (99%) |
| **SA2** | 688 | *A. thaliana* calmodulin-binding receptor-like kinase 2 (CRCK2) | NM116255 | (72%) |
| **SA3** | 772 | Polyribonucleotide nucleotidyltransferase | CP000323 | (92%) |
| **SA4** | 263 | Chromosomal replication initiator protein DnaA | CP000653 | (90%) |

**Table 6 Result analysis of DEGs through KOBAS; KO Based Annotation System for the pathway identification**

| Sequence identifier | KO term | KO definition | Rank | E-value | Score | Identity (%) | Blast hit |
|---|---|---|---|---|---|---|---|
| SA3 | K00962 | Polyribonucleotide nucleotidyltransferase | 1 | 1e-111 | 404.0 | 96.64 | Pcr. Pcryo 0080 |

Each row corresponds to a query DNA or protein input by the user. The first column contains sequence identifier extracted from the input. The second column contains the assigned KO terms hyperlinked to detailed description in KEGG. The third column contains KO term definition that this protein sequence belongs to this available protein in this program. The fourth to seventh columns shows the rank, e-value, score and identity of the BLAST hit. The last column contains the gene ID of the hit hyperlinked to the KEGG GENES dataset database.

into *E. coli* 10b. The transformation mixture was then spread on 100 mg/ml LB ampicillin petri plates after incubation for one h at 37°C. Plates were incubated overnight at 37°C and the next day colonies were cultured in LB containing ampicillin and placed overnight in a shaking water bath at 37°C. Plasmid isolation from cultures was performed by miniprep method and recombinant clone was confirmed by digestion with *Eco*RI and *Hin*dIII. The resultant recombinant clone was named pSAβC135S.

### Transfer of expression cassette to binary vector and transformation of Agrobacterium tumefaciens

pSAβC135S and pGreen0029 were restricted with *Xho*I and *Xba*I endonuclease, ethanol precipitated and ligated at 16°C for 18 h. This was used for transformation into *E. coli* and colonies were confirmed by restriction analysis. Both pGreen0029 and pGreen0029 containing the expression cassette were transformed into *Agrobacterium tumefaciens* strain (GV 3101) by electroporation. The transformation mixture was then spread on LB medium plates containing 50 μg/ml of kanamycin, 25 μg/ml of rifampicin and 100 μg/ml tetracycline antibiotics, after a one hour incubation at 28° C. Plates were incubated at 28°C until colonies appeared. After 48 hours, colonies were grown in LB liquid medium containing 50 μg/ml of kanamycin, 25 μg/ml of rifampicin and 100 μg/ml tetracycline, and placed at 28°C for 48 h. The transformants were confirmed by PCR analysis using the primers ChβC135S (F)/ChβC135S(R).

### Agroinfiltration of plants

*Agrobacterium* cultures were grown at 28°C for 48 h in liquid LB medium containing 50 μg/ml of kanamycin and 25 μg/ml of rifampicin. The bacterial cells were pelleted at 4000 rpm for 10 min at 20°C and resuspended in 10 mM $MgCl_2$ and 150 μg of acetosyringone per ml. After a three hour incubation cells were infiltrated into young, fully expanded leaves of 4 week- old *N. tabacum* plants using a 5 ml syringe.

### Isolation of messenger RNA and cDNA synthesis

Infiltrated of *N. tabacum* leaves infiltrated with pGreen0029 and pGreen0029 containing the βC1 expression cassette were collected two and four days after inoculation in liquid nitrogen. Total RNA was extracted using Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. The integrity and purity of total RNA isolated from infected leaf samples was assessed by electrophoresis on 1% agarose gels. The messenger RNA was isolated from total RNA using oligo (dT) cellulose columns (MRC, USA) according to the manufacturer's instructions. The loaded columns were washed with binding buffer and mRNA was eluted. The eluted mRNA was precipitated and dissolved in DEPC treated water. Messenger RNA resulting from two and four days post infiltration samples were reverse transcribed to cDNA using Revert Aid H- First Strand cDNA synthesis kit, (Fermentas, USA). Three reverse transcription reactions were carried out for each mRNA using three different anchored (T11M) primers (where M may be G, C or A). The products of reverse transcriptions (cDNA) were stored at -20° C for differential display PCR amplifications.

### Differential display analysis

PCR amplification of each cDNA (synthesized from mRNA isolated from two and four days post inoculation samples) was carried out in combination with one of the three anchored primers and one of the twenty arbitrary

**Table 7 Summary of purine and pyrimidine metabolic pathways of Polynucleotide nucleotidyl transferase**

| Query gene | Pathway | Count and ratio | p-value | q-value | Web site |
|---|---|---|---|---|---|
| SA3 | Pyrimidine metabolism | **1/100% 44/1.53%** | 0.0153417015342 | 0.023709902371 | http://www.genome.jp/kegg/catalog/org_list.html<br>http://kobas.cbi.pku.edu.cn/help.do<br>http://kobas.cbi.pku.edu.cn<br>http://www.genome.jp/ |
| SA3 | Purine metabolism | **1/100% 68/2.37%** | 0.023709902371 | 0.023709902371 | |

Table 4 described the first column shows the name of the pathway. The second column lists the number and percentage of input genes or proteins involved in the pathway (top red in color) and the number and percentage of background genes or proteins involved in the pathway (bottom green in color). The third and fourth columns list the p-value and q-value of the statistical significance, respectively. Purine and pyrimidine metabolic pathways of (SA3) polynucleotide nucleotidyltransferase that is an RNA binding protein and involved in RNA degradation.

primers of the B-Series (as indicated in Table 1), providing 60 combinations in case of four days post inoculation and 29 combinations in the case of two days post inoculation. PCR was carried out in a final reaction volume of 50 μl containing 2.5 μl (100 ng/μl and 10 ng/μl) of first strand cDNA, 5 μl of 10× PCR buffer, 4 μl $MgCl_2$ (25 mM), 1 μl of dNTPs (10 mM each), 2 μl of anchord primer (250 ng/μl), 8 μl of arbitrary primer (100 ng/μl), 0.5 μl of *Taq* DNA Polymerase (5 U/μl; Fermentas, USA), 27 μl double distilled $H_2O$. The PCR amplification protocol included first cycle at 94°C for 4 min followed by 45 cycles of 36°C for 2 min, 72°C for 1 min, 94°C for 1 min; and a final extension step at 72°C for 10 min. The amplified PCR products were resolved on 1% agarose gel and stained with ethidium bromide.

### Cloning and sequencing of differentially expressed genes (DEGs)

The differentially expressed bands were excised from the gel and extracted by QIAGEN gel extraction kit and DNA extraction kit (MBI, Fermentas). The eluted bands were ligated into pTZ57RT, and transformed into *E. coli* TOP 10 by the heat shocked method. Plasmid DNA was isolated using the miniprep method and clones were confirmed by restriction analysis using *Eco*RI and *Pst*I restriction enzymes. Purified clones were sequenced using M13 (-20) forward and M13 (-26) reverse primers and BigDye terminator v 3.1 ABI Prism 310 Genetic analyzer (Applied Biosystems, USA) as decribed by the manufacturer. Sequence information was stored, assembled and analysed using the Lasergene sequence

**Table 8 After two days differentially expressed genes (DEGs) primer sequences for quantitative real time PCR**

| Name of genes | Sequences of primers | MERS |
|---|---|---|
| **Primer sequences two days post inoculation of βC1 of ChLCB for Q-RTPCR analysis** | | |
| **SAA** | SAA F: GAGACCCGGGATGTCCTGGCAAGAAAGCAT | **(30 MERS)** |
| | SAAQPCR:AATTACAAAAGAGCCCCTAAATCCCTAAGC | **(30 MERS)** |
| | SAA F2: GGAGAGGGCAACCGATGA | **(18 MERS)** |
| | SAA QPCR2: CCCCTAAATCCCTAAGC | **(17 MERS)** |
| | SAA F3: GGGACGATCGCCGGCGCCGG | **(20 MERS)** |
| | SAA QPCR3: TCACTACCCACCGTATC | **(17 MERS)** |
| **SAB** | SAB F: AATCCCCGGGATGTATGCTCCGAATCCCGC | **(30 MERS)** |
| | SABQPCR:CATAGTGATGTCGAAAGCAAAAGTAGGGCC | **(30 MERS)** |
| | SAB F2: GTATGCTCCGAATCCCG | **(17 MERS)** |
| | SAB QPCR2: CAAAAGTAGGGCCTTCC | **(17 MERS)** |
| | SAB F3: CCAGCTAAGGGAGGAATC | **(18 MERS)** |
| | SAB QPCR3: GGCCTTCCACTGTCTTCCTG | **(20 MERS)** |
| **SAC** | SAC F: TCCCCCCGGGATGTTTCAGGTTCACATGAA | **(30 MERS)** |
| | SACQPCR:TAGGCTATAGGTGGGGGACAATGTAGACTG | **(30 MERS)** |
| | SAC F2: CACAACACGACTCCCTAC | **(18 MERS)** |
| | SAC QPCR2: GAAGTTGGGCCCACCTG | **(17 MERS)** |
| | SAC F3: CTCCACGAGTCTTCATCCCC | **(20 MERS)** |
| | SAC QPCR3: CCGAGATCGAGAGCTTTC | **(18 MERS)** |
| **SAD** | SA D F; GGTGCCCGGGATGGCAGATCAGTGGAGTTG | **(30 MERS)** |
| | SADQPCR: GATTAGGTTCCCGTAGATAGATGCATAACC | **(30 MERS)** |
| | SAD F2: AAGTTCTAATTCGGAGGG | **(18 MERS)** |
| | SAD QPCR2: TAGATAGATGCATAA | **(17 MERS)** |
| | SAD F3: GTTAGCTTACTTAAACAG | **(20 MERS)** |
| | SAD QPCR3: TAGATGCATAACC | **(17 MERS)** |

**Table 9 After four days differentially expressed genes (DEGs) primer sequences for quantitative real time PCR**

| Name of genes | Sequences of primers | MERS |
|---|---|---|
| **Primer sequences four days post inoculation of βC1 of ChLCB for Q-RTPCR analysis** | | |
| **SA1** | SA1 F: GTCACCCGGGATGTGACGCCGACGGTCAAT | **(30 MERS)** |
| | SA1 QPCR: GGGCCGCACCATGGTCCTGCTGACTTACCG | **(30 MERS)** |
| | SA1 F2: GACGGTCAATCCATGTAT | **(18 MERS)** |
| | SA1 QPCR2: GGTGTCAGGAGACCCCTTCCA | **(17 MERS)** |
| | SA1 F3: GGTAGAGCCCCAGTCTTCCA | **(20 MERS)** |
| | SA1 QPCR3: GCACCCGCCCAACTCCACGG | **(17 MERS)** |
| **SA2** | SA2 F: AATACCCGGGATGATAAACATTTGGGGG | **(30 MERS)** |
| | SA2 QPCR: CCAATGTCTAGTCTTGATGCAAAATCAA | **(30 MERS)** |
| | SA2 F2: CTAGTAAAGTTTTATGGATTCTTGGA | **(17 MERS)** |
| | SA2 QPCR2: ATGGATAATAGGGTGATCAGT | **(17 MERS)** |
| | SA2 F3: CACTTGGACTGTGGTCCTG | **(18 MERS)** |
| | SA2 QPCR3: GTCAGCCACCTTAGCTCG | **(20 MERS)** |
| **SA3** | SA3 F: CGCGCCCGGGATGCATCTAGATTGTCCACA | **(30 MERS)** |
| | SA3 QPCR: TCAATCAGACGCGAGGTTAAGGTTTCAGAC | **(30 MERS)** |
| | SA3 F2: GAAGGCTATGTAAACGAG | **(18 MERS)** |
| | SA3 QPCR2: GCTCTTCAAGGGTCGGGTTCAG | **(17 MERS)** |
| | SA3 F3: GACTTGGTCGTCGCTGGTA | **(20 MERS)** |
| | SA3 QPCR3: GCTTGATCGCGTACAGG | **(18 MERS)** |
| **SA4** | SA4 F: GCGACCCGGGAtGCATCTAGATTTGGGGGA | **(30 MERS)** |
| | SA4 QPCR: AGAAACAGAAGATCTCTGGCTCAGTTTAGG | **(30 MERS)** |
| | SA4 F2: TTCATGATTGTTGGCGCAC | **(18 MERS)** |
| | SA4 QPCR2: CTGATCTTCCTGTGGA | **(17 MERS)** |
| | SA4 F3: CGGCATGACCCTGTGTAA | **(20 MERS)** |
| | SA4 QPCR: GGGGGACTCGCGCCAGG | **(17 MERS)** |

analysis package (DNAStar Inc., Madison, WI, USA) running on an IBM compatible PC.

### Analysis of DEGs using NCBI, KOBAS and RT-PCR

The nucleotide sequences were analyzed using BLAST; for blastn and blastx algorithms in NCBI. Clusters of orthologus group of proteins were identified at NCBI http://www.ncbi.nlm.nih.gov/Blast.cgi and KEGG orthology http://www.genome.jp/. Real time quantitative PCR was performed to analyse expression of DEGs in relation to a reference gene and the calibrators at a constant level of fluorescence. These were calculated with Delta Delta (Ct), Livak and the Pfaffi mathematical models of quantitative real time PCR method [57,58]. For RT-PCR each sample was used in triplicate and the experiment was repeated three times to confirm the reproducibility of result. The sequences of RT-PCR primers are shown in Table 8 and 9.

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#### Authors' contributions

SA conducted all the experimental work and drafted the manuscript. AB and IA helped in the RT-PCR and DD-PCR analysis. SM and RWB together designed the experiments. IA and SM had proof-read and finalized the manuscript. All authors read and approved the final manuscript.

#### Competing interests

The authors declare that they have no competing interests.

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