

Tomato cultivar tolerant to *Tomato leaf curl New Delhi virus* infection induces virus-specific short interfering RNA accumulation and defence-associated host gene expression

PRANAV PANKAJ SAHU¹, NEERAJ K. RAI¹, SUPRIYA CHAKRABORTY², MAJOR SINGH³, PRASANNA H. CHANDRAPPA³, BANDARUPALLI RAMESH⁴, DEBASIS CHATTOPADHYAY¹ AND MANOJ PRASAD^{1,*}

¹National Institute of Plant Genome Research, Aruna Asaf Ali Marg, New Delhi-110067, India

²School of Life Sciences, Jawaharlal Nehru University, New Delhi-110067, India

³Indian Institute of Vegetable Research, Gandhinagar, Varanasi-221005, India

⁴Department of Genetics and Plant Breeding, Ch. Charan Singh University, Meerut-250004, India

SUMMARY

Tomato leaf curl New Delhi virus (ToLCNDV) infection causes significant yield loss in tomato. The availability of a conventional tolerance source against this virus is limited in tomato. To understand the molecular mechanism of virus tolerance in tomato, the abundance of viral genomic replicative intermediate molecules and virus-directed short interfering RNAs (siRNAs) by the host plant in a naturally tolerant cultivar H-88-78-1 and a susceptible cultivar Punjab Chhuhara at different time points after agroinfection was studied. We report that less abundance of viral replicative intermediate in the tolerant cultivar may have a correlation with a relatively higher accumulation of virus-specific siRNAs. To study defence-related host gene expression in response to ToLCNDV infection, the suppression subtractive hybridization technique was used. A library was prepared from tolerant cultivar H-88-78-1 between ToLCNDV-inoculated and *Agrobacterium* mock-inoculated plants of this cultivar at 21 days post-inoculation (dpi). A total of 106 nonredundant transcripts was identified and classified into 12 different categories according to their putative functions. By reverse Northern analysis and quantitative real-time polymerase chain reaction (qRT-PCR), we identified the differential expression pattern of 106 transcripts, 34 of which were up-regulated (>2.5-fold induction). Of these, eight transcripts showed more than four fold induction. qRT-PCR analysis was carried out to obtain comparative expression profiling of these eight transcripts between Punjab Chhuhara and H-88-78-1 on ToLCNDV infection. The expression patterns of these transcripts showed a significant increase in differential expression in the tolerant cultivar, mostly at 14 and 21 dpi, in comparison with that in the susceptible cultivar, as analysed by

qRT-PCR. The probable direct and indirect relationship of siRNA accumulation and up-regulated transcripts with the ToLCNDV tolerance mechanism is discussed.

INTRODUCTION

Plants are routinely attacked by a spectrum of parasites, such as viruses, bacteria, fungi, nematodes and insects. Unlike animals, plants are sessile and lack a somatically adaptive immune system in their response to pathogens. Instead, plants have evolved various multifaceted mechanisms to protect themselves from various biotic and abiotic challenges (Thordal-Christian, 2003). A large number of crop plants are susceptible to infection by viruses. Geminiviruses are an emerging group of plant viruses that affect horticultural crops in tropical and subtropical areas throughout the world. Tomato leaf curl disease (ToLCD) is one of the most important constraints to tomato production in the tropics and subtropics, particularly in South and Southeast Asia (Chakraborty, 2008; Czosnek and Laterrot, 1997; Green and Kalloo, 1994; Saikia and Muniyappa, 1989; Vasudeva and Samraj, 1948). The disease is caused by strains of *Tomato leaf curl New Delhi virus* (ToLCNDV), a species of the genus *Begomovirus*, family *Geminiviridae*, and is transmitted by whiteflies (*Bemisia tabaci* Genn.). The limited availability of a conventional resistance source has restricted the efforts to develop a cultivar with durable resistance. The majority of the begomoviruses within the Old World are monopartite in nature and often associated with satellite DNA- β molecules (Nawaz-ul-Rehman *et al.*, 2009). However, bipartite begomoviruses, such as ToLCNDV (possessing DNA-A and DNA-B of about 2.5–2.7 kb in size), are predominant in major tomato-growing areas of the country (Chakraborty, 2008). DNA-A encodes the components necessary

*Correspondence: Email: manoj_prasad@nipgr.res.in

for replication and DNA-B encodes proteins for systemic movement inside the host. The viral genome multiplies by a combination of rolling circle and recombination-dependent replication in the nucleus via a double-stranded DNA intermediate (Gutierrez, 1999; Jeske *et al.*, 2001; Preiss and Jeske, 2003). At least five species of *Tomato leaf curl virus* (ToLCV) have been reported in southern India (Kirthi *et al.*, 2002), where ToLCD incidence in the summer season (February to June) in susceptible varieties increases rapidly to 100% and yield losses often exceed 90% (Saikia and Muniyappa, 1989). In northern India, ToLCNDV is the predominant species, resulting in severe ToLCD, either alone or in combination with other tomato-infecting begomoviruses (Chakraborty, 2008).

Interactions between plants and pathogens induce a series of defence responses (Hammond-Kosack and Jones, 1996). When a plant and a pathogen come into contact, close communications occur between the two organisms (Hammond-Kosack and Jones, 2000). Plants are adapted to detect the presence of pathogens and to respond with antimicrobial defences and other stress responses. Systemic infection of plants by virus requires modifications that allow viral replication and movement. This is associated with the suppression of host gene defence responses and changes in host gene expression. It has been shown that the induction of these mechanisms involves a complex network of signal perception, amplification and transduction, in which several molecules and defence-related genes participate (Xiong *et al.*, 2001). Several studies have investigated the genes expressed during begomovirus–host interaction. In *Arabidopsis*, numerous defence-associated genes have been identified and have shown to be coordinately regulated in response to infection with various viruses (Whitham *et al.*, 2003). During these interactions, the plant defence system is strictly regulated which, in turn, determines the outcome (McDowell and Dangle, 2000). Undoubtedly, these diverse and complex interactions are a product of many factors, including differences in signal transduction between the interacting organisms and the relative dominance of the pathway involved (Takemoto and Hardham, 2004).

Begomoviruses have been shown to induce and to become the target of post-transcriptional gene silencing (PTGS) in plants (Akbergenov *et al.*, 2006; Chellappan *et al.*, 2004; Lucioli *et al.*, 2003). Symptom recovery in infected plants over time is correlated with the accumulation of short interfering RNAs (siRNAs) targeted against specific viruses (Rodriguez-Negrete *et al.*, 2008). siRNA-mediated gene silencing has become an important method for the analysis of gene functions in eukaryotes and, at the same time, holds promise for the development of new approaches aimed at controlling plant viruses. The molecular basis of disease development following virus infection has been the subject of intense investigation (Vanitharani *et al.*, 2005). The ability to silence viral genes by the generation of cellular double-stranded RNA (dsRNA) targeted against viral genomes

has resulted in the suppression of virus infection in animal as well as plant systems (Ding *et al.*, 2004).

The subtractive complementary DNA (cDNA) hybridization technique is a powerful approach to gain preliminary insights by identifying and isolating cDNA of differentially expressed genes. Suppressive subtractive hybridization (SSH) is used to selectively amplify target cDNA fragments (differentially expressed) and simultaneously suppress the amplification of nontarget DNA. The method is based on the suppression polymerase chain reaction (PCR) effect. In order to identify the molecular mechanisms underlying tolerance or resistance to virus infections, a strategy combining SSH with reverse Northern analysis and quantitative real-time polymerase chain reaction (qRT-PCR) was used to analyse the difference in gene expression between tolerant and susceptible tomato cultivars, namely H-88-78-1 and Punjab Chuhara, respectively. To understand the molecular regulation of these processes, the relevant subsets of differentially expressed transcripts of interest were identified, cloned and studied in detail. Those transcripts expressed in the tolerant cultivar alone may act as stress sensors, transcriptional activators or signal transduction pathway components, and thus may behave as dominating factors for resistance towards viral infection. We identified a series of transcripts that may exhibit viral tolerance inducibility, including stress response factors, transporters, transcription factors and transcripts encoding proteins of unknown functions. In the present investigation, we studied the tolerant phenotypic character of tomato cultivar H-88-78-1 in terms of viral DNA and siRNA accumulation and host response to ToLCNDV infection.

RESULTS AND DISCUSSION

Infectivity analysis for ToLCNDV tolerance in tomato cultivars

We screened seven tomato cultivars for tolerance and susceptibility to ToLCNDV infection under glasshouse conditions. Scoring was performed on the basis of the percentage infectivity at 21 days post-inoculation (dpi), and was subsequently classified as tolerant (T), moderately tolerant (MT), susceptible (S) and highly susceptible (HS) (Table 1). A total of 75 plants from three independent experiments was tested for infectivity analysis for each cultivar. Leaf curl symptoms started to appear in the cultivar Punjab Chuhara (highly susceptible to ToLCNDV) within 7 dpi, whereas, in the highly resistant cultivar H-88-78-1, symptom initiation started at 15 dpi (Table 1). We scored the number of plants showing symptoms at 21 dpi (Fig. 1). The highest infectivity (92%) was observed in cultivar Punjab Chuhara, followed by 86.7% in 155B, both of which were grouped as highly susceptible cultivars (Table 1). Cultivar H-86 was categorized as susceptible, with 58.6% of the inoculated plants showing symp-

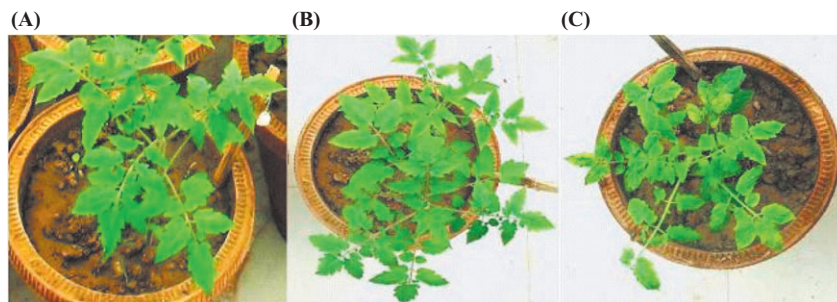


Fig. 1 Phenotypic expression of tomato cultivars at 21 days post-inoculation (dpi). (A) H-88-78-1 inoculated with pCAMBIA2301 as mock. (B) H-88-78-1 inoculated with *Tomato leaf curl New Delhi virus* (ToLCNDV). (C) Punjab Chuhara inoculated with ToLCNDV.

Table 1 Infectivity of *Tomato leaf curl New Delhi virus* in selected tomato cultivars by *Agrobacterium*-mediated inoculation.

Cultivar	Plants infected/inoculated	First symptom appearance (dpi)	Symptom severity*	Overall gradet
LA1777	9/75	12	+	T
H-88-78-1	7/75	15	+	T
TLBRH-5	29/75	10	++	MT
TLBRH-6	27/75	10	++	MT
H-86	44/75	9	+++	S
Punjab Chuhara	69/75	7	++++	HS
155B	65/75	7	++++	HS

Observations were made at 21 days post-inoculation (dpi).

*+, Least severe; ++, moderately severe; +++, severe; +++++, highly severe. †T, Tolerant (1–20%); MT, moderately tolerant (20.1–40%); S, susceptible (40.1–60%); HS, highly susceptible (60.1–100%).

toms, whereas hybrids TLBRH-5 and TLBRH-6 were considered to be moderately tolerant with 38.7% and 36% infection, respectively. Two cultivars, H-88-78-1 and LA1777, showed lower infectivity (9.3% and 12%, respectively), and hence were grouped as tolerant cultivars (Table 1). Southern blotting of infected tomato genomic DNA with a part of ToLCNDV DNA-A as probe showed a 2.4-fold higher accumulation of viral replicative intermediate in Punjab Chuhara in comparison with that in H-88-78-1 at 21 dpi (Fig. 2A,B). Hence, H-88-78-1 was selected as a tolerant cultivar for further studies.

Accumulation of siRNA in tolerant cultivars

To study the molecular mechanism of tolerance in these tomato cultivars, the accumulation of viral gene-specific siRNAs in the leaf tissues of H-88-78-1 and Punjab Chuhara was studied at different time points. To determine the association of antiviral defence with PTGS in terms of virus-specific siRNA accumulation, total RNA was analysed by polyacrylamide gel electrophoresis (PAGE) to resolve low-molecular-weight RNAs. Northern blots with replication-associated protein (Rep) gene as probe detected the accumulation of 21–23-nucleotide-long RNA products in virus-infected samples, indicating that the ToLCNDV-infected cultivar H-88-78-1 was able to induce PTGS with the

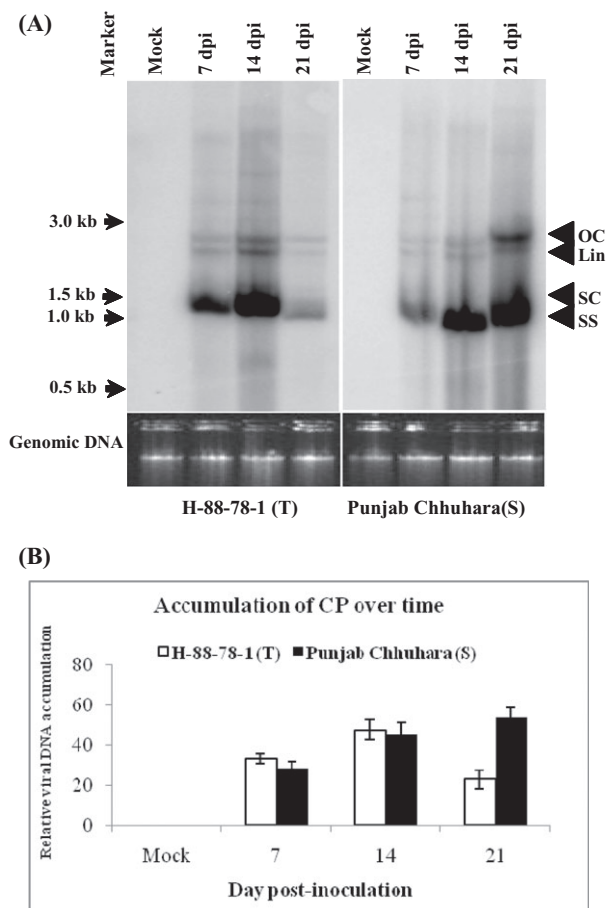


Fig. 2 Infectivity analysis on tolerant (H-88-78-1; T) and susceptible (Punjab Chuhara; S) cultivars of tomato. (A) Southern blot of tomato genomic DNA hybridized with *Tomato leaf curl New Delhi virus* (ToLCNDV) coat protein (CP) gene as probe. (B) Relative accumulation of viral DNA in ToLCNDV-infected tolerant cultivar H-88-78-1 and susceptible cultivar Punjab Chuhara at different time points. Viral replicative forms are indicated as open circular (OC), linear (Lin), supercoiled (SC) and single strand (SS). Bars indicate the standard deviations (\pm SD).

production of virus-specific siRNAs (Fig. 3A). The blot was quantified for siRNA accumulation, and it was found that the amount of siRNAs increased gradually (>85-fold at 21 dpi) with time in H-88-78-1 (Fig. 3A,B). In infected cultivar H-88-78-1, virus-

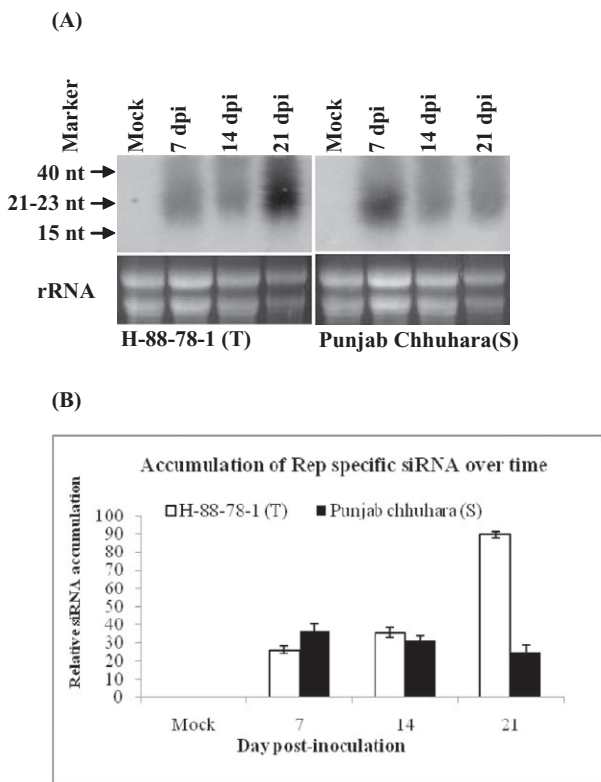


Fig. 3 Identification of *Tomato leaf curl New Delhi virus* (ToLCNDV)-derived short interfering RNA (siRNA) in tolerant (H-88-78-1; T) and susceptible (Punjab Chhuhara; S) cultivars of tomato. (A) Northern hybridization showing the accumulation of virus-derived siRNAs. (B) Identification of replication-associated protein (Rep)-specific siRNA accumulation in ToLCNDV-infected tolerant cultivar H-88-78-1 and susceptible cultivar Punjab Chhuhara at different time points. A plant infected with *Agrobacterium tumefaciens* harbouring pCAMBIA2301 alone was used as mock. Total RNA was separated by 15% polyacrylamide gel electrophoresis and hybridized with the ToLCNDV Rep gene fragment as probe. The sizes of the standard oligonucleotides and siRNAs are indicated. Bars indicate the standard deviations (\pm SD). Ethidium bromide-stained ribosomal RNA is shown for equivalent loading.

derived siRNA accumulation was 26-fold at 7 dpi, 36-fold at 14 dpi and reached a maximum of 90-fold at 21 dpi (Fig. 3B). However, in the cultivar Punjab Chhuhara, a contrasting pattern in the rate of siRNA accumulation was observed. In Punjab Chhuhara, siRNA accumulation started at 7 dpi (43-fold), when it was more abundant when compared with the tolerant cultivar; it then decreased at 14 dpi (32-fold) and 21 dpi (25-fold) (Fig. 3B). The increased level of siRNA at later times in cultivar H-88-78-1 suggests that this accumulation may have a possible correlation with the lower level of viral replication in this cultivar. The induction of siRNA has been shown to down-regulate viral DNA accumulation and gene expression in plant cells (Vanitharani *et al.*, 2003). The accumulation of virus-derived siRNAs has been reported in local and systemically infected leaf tissue of virus-

infected plants (Moissiard and Voinnet, 2004; Szittyá *et al.*, 2002), demonstrating the activation of virus-induced gene silencing (VIGS), which results in reduced accumulation of the invading virus (Szittyá *et al.*, 2002). In our experiment, Southern blot analysis also indicated less accumulation of viral DNA-A in the tolerant cultivar at 21 dpi (Fig. 2A,B). However, the intensity and efficacy of virus-induced PTGS varies with intrinsic features of the viral genome and its interaction with the host (Moissiard and Voinnet, 2004; Voinnet, 2001, 2005; Waterhouse *et al.*, 2001). Mechanisms other than the mere accumulation of siRNA are also likely to play a role in the natural resistance against virus infection, as transgene-mediated siRNAs are also produced in susceptible plants (Ribeiro *et al.*, 2007).

Identification and classification of ToLCNDV-responsive genes in the tolerant cultivar

A total of 400 clones was randomly picked from the forward subtracted library. After single-pass sequencing and vector sequence removal, 259 expressed sequence tag (EST) sequences were obtained, 106 of which were nonredundant. All of these 106 clones were functionally annotated by BLASTX against the GENBANK nonredundant EST databases, and subsequently classified into 12 functional categories according to their putative function (Table 2). Transcripts assigned to the metabolism category accounted for the largest (32.07%) contribution, followed by photosynthetic transcripts (14.15%). Transcripts were also involved in other processes, including cell cycle/DNA and RNA processing (4.71%), signalling/receptor function (4.71%), hormone/hormonal regulation (4.71%), abiotic stress (1%), cell development/housekeeping (7.54%), cellular transport (6.60%), transcription/translational factor (5.66%), defence response (3.77%), protein degradation (5.66%) and unknown function (9.43%) (Table 2). Our study listed the abundance of transcripts in the host plant in response to ToLCNDV infection. Similar studies have been reported on *Tomato mottle taino virus* (ToMoTV) and *Cucumber mosaic virus* (CMV) to identify the up-regulated host transcripts during infection (Collazo *et al.*, 2005; Ruiz-Medrano *et al.*, 2007).

Identification of differentially expressed clones by reverse Northern analysis

The effective signal intensities of the spots were calculated by subtracting the normalized intensity of the negative control. Reverse Northern hybridization analysis revealed that, of the 106 transcripts, 34 (32.07%) were up-regulated (more than 2.5-fold induction) in response to ToLCNDV infection (Table 2). Of these, eight transcripts showed more than four fold induction after ToLCNDV infection in comparison with control at 21 dpi (Fig. 4A, B). These identified transcripts were involved in the defence

Table 2 Categorization of transcripts expressed in tolerant tomato cv. H-88-78-1 after *Tomato leaf curl New Delhi virus* (ToLCNDV) infection according to their putative function and fold induction.

GENBANK accession ID	Similarity of transcripts	GENBANK match	Organism	Obtained sequence length (bp)†	E-value	Fold induction (± SD)*
CELL CYCLE AND DNA/RNA PROCESSING						
GR979494	RNA polymerase-associated protein LEO1	EEF47773.1	<i>Ricinus communis</i>	808	3.00E-60	3.27 (± 0.33)
GR979429	Histone h2b, putative	EEF29752.1	<i>R. communis</i>	337	1.00E-19	1.43 (± 0.89)
GR979472	Dead box ATP-dependent RNA helicase	EEF41818.1	<i>R. communis</i>	539	2.00E-48	3.37 (± 1.24)
GR979481	Putative DNA2-NAM7 helicase family protein	AAL31652.1	<i>Oryza sativa</i>	833	6.00E-121	3.98 (± 0.93)
GR979485	FtsH-like protein Pftf precursor	AAD17230.1	<i>Nicotiana tabacum</i>	469	5.00E-42	-1.89 (± 0.37)
SIGNALLING /RECEPTOR						
GR979482	Serine/threonine protein kinase PBS1†	EEF43813.1	<i>R. communis</i>	607	4.00E-65	4.01 (± 0.79)
GR979493	Signal recognition particle 19 kDa protein	EEF43168.1	<i>R. communis</i>	496	1.00E-26	2.34 (± 0.97)
GR979435	Receptor protein kinase	EEF50514.1	<i>R. communis</i>	513	1.00E-52	1.54 (± 0.41)
GR979412	Chloroplast N receptor-interacting protein 1	ACA79924.1	<i>Nicotiana benthamiana</i>	309	2.00E-31	-1.82 (± 0.39)
GR979457	ADP-ribosylation factor 1	ACG31280.1	<i>Zea mays</i>	288	1.00E-49	3.07 (± 1.73)
HORMONE/HORMONAL REGULATION						
GR979487	Gibberellin-regulated protein 1†	EEF35557.1	<i>R. communis</i>	618	6.00E-11	4.15 (± 0.75)
GR979477	Auxin influx transport protein	ABN81352.1	<i>Casuarina glauca</i>	679	4.00E-123	2.26 (± 0.83)
GR979484	Indole-3-acetic acid-amido synthetase GH3	EEF28642.1	<i>R. communis</i>	601	3.00E-17	2.01 (± 0.13)
GR979403	Similar to IBR3	refNP_187337.2	<i>Vitis vinifera</i>	850	2.00E-116	2.09 (± 0.93)
GR979430	Cytokinin binding protein CBP57	dbjBAA03710.1	<i>Nicotiana sylvestris</i>	214	9.00E-30	1.06 (± 0.42)
ABIOTIC STRESS						
GR979463	ERD15	ABB89735.1	<i>Capsicum annum</i>	493	2.00E-60	-2.23 (± 0.13)
CELL DEVELOPMENT/HOUSEKEEPING						
GR979431	60S ribosomal protein L13	AAQ96375.1	<i>Solanum brevidens</i>	570	1.00E-90	1.23 (± 0.76)
GR979459	Similar to HSC70-1	ABX26256.1	<i>Panax quinquefolius</i>	258	9.00E-38	1.75 (± 0.57)
GR979461	Actin	ACJ04738.1	<i>Sedum alfredii</i>	479	8.00E-82	1.03 (± 0.56)
GR979434	α-Tubulin	embCAD13178.1	<i>N. tabacum</i>	502	4.00E-82	1.02 (± 0.36)
GR979424	Cell wall protein	embCAA54561.1	<i>Solanum lycopersicum</i>	439	3.00E-09	1.37 (± 0.41)
GR979420	Ribosome biogenesis protein nop10	EEF32551.1	<i>R. communis</i>	232	3.00E-26	1.01 (± 0.31)
GR979417	Senescence-associated protein	AAZ23261.1	<i>N. tabacum</i>	308	1.00E-36	1.57 (± 0.83)
GR979492	Xyloglucan endotransglycosylase	AAZ08349.1	<i>S. lycopersicum</i>	386	2.00E-73	-1.87 (± 0.91)
METABOLISM						
GR979471	Phosphoglycerate kinase precursor	AAC26785.1	<i>Solanum tuberosum</i>	503	5.00E-85	1.48 (± 0.77)
GR979401	Aminomethyltransferase	spP54260.1	<i>S. tuberosum</i>	462	1.00E-81	1.98 (± 0.39)
GR979392	C-4 sterol methyl oxidase 2	AAQ83692.1	<i>N. benthamiana</i>	267	2.00E-30	2.12 (± 0.93)
GR979425	S-Adenosylmethionine decarboxylase	ABQ42184.1	<i>S. lycopersicum</i>	500	6.00E-26	2.33 (± 0.67)
GR979409	Plastidic aldolase NPALDP1	dbjBAA77604.1	<i>Nepenthes paniculata</i>	352	1.00E-30	1.93 (± 0.13)
GR979456	Triose phosphate isomerase	ABB02628.1	<i>S. tuberosum</i>	371	1.00E-60	2.63 (± 0.29)
GR979444	Glycine hydroxymethyltransferase	spP50433.1	<i>S. tuberosum</i>	845	6.00E-151	2.29 (± 0.31)
GR979427	Glyoxysomal malate dehydrogenase	AAU29200.1	<i>S. lycopersicum</i>	790	9.00E-140	1.17 (± 0.69)
GR979447	Plastid glutamine synthetase GS2	AAG40236.2	<i>S. tuberosum</i>	217	1.00E-34	2.23 (± 0.72)
GR979399	Geranylgeranyl reductase	embCAA07683.1	<i>N. tabacum</i>	399	5.00E-71	1.23 (± 0.77)
GR979448	Serine-pyruvate aminotransferase, putative	EEF31258.1	<i>R. communis</i>	447	8.00E-68	1.14 (± 0.47)
GR979449	Hydroxypyruvate reductase	dbjBAB44155.1	<i>R. communis</i>	293	3.00E-46	-1.19 (± 0.82)
GR979450	Catalase	AAR14052.2	<i>S. tuberosum</i>	744	4.00E-148	-1.87 (± 0.59)
GR979468	Alanine aminotransferase 2	refNP_0011151209.1	<i>Z. mays</i>	542	1.00E-36	1.59 (± 0.87)
GR979466	Phosphoribulokinase	refNP_001149223.1	<i>Z. mays</i>	716	2.00E-112	-1.22 (± 0.86)
GR979440	Precursor of carboxylase h-protein 4	refXP_002326710.1	<i>Populus trichocarpa</i>	449	2.00E-64	1.04 (± 0.34)
GR979453	Aldolase	AAA33643.1	<i>Pisum sativum</i>	230	3.00E-35	2.59 (± 0.11)
GR979421	Catalase isozyme	ABY21246.1	<i>S. tuberosum</i>	196	1.00E-30	1.15 (± 0.41)
GR979489	β-Mannosidase	AAL37714.1	<i>S. lycopersicum</i>	519	4.00E-58	-1.12 (± 0.69)
GR979452	Glyceraldehyde-3-phosphate dehydrogenase	ABW89104.1	<i>Helianthus annuus</i>	576	6.00E-75	2.67 (± 0.67)
GR979476	PAPS-reductase-like protein	AAB05871.2	<i>Catharanthus roseus</i>	532	2.00E-35	2.23 (± 0.46)
GR979478	FAD5 (FATTY ACID DESATURASE 5)	refNP_566529.1	<i>Arabidopsis thaliana</i>	375	3.00E-56	2.11 (± 0.63)
GR979483	Acyl carrier protein	EEF35894.1	<i>A. thaliana</i>	790	4.00E-42	1.86 (± 0.49)
GR979396	Glycosyltransferase	refXP_002323701.1	<i>P. trichocarpa</i>	561	6.00E-57	1.31 (± 0.34)
GR979469	Benzoyl coenzyme A	AAT68601.1	<i>Petunia hybrida</i>	269	2.00E-38	-1.07 (± 0.63)
GR979389	Glycine dehydrogenase	spO49954.1	<i>S. tuberosum</i>	407	8.00E-21	2.78 (± 1.09)
GR979405	5-Phosphoribosyl-1-pyrophosphate amidotransferase	AAR06289.1	<i>N. tabacum</i>	357	3.00E-37	1.36 (± 0.25)
GR979410	Carbonic anhydrase	embCAH60891.1	<i>S. lycopersicum</i>	468	2.00E-62	1.91 (± 0.23)
GR979467	Inorganic pyrophosphatase	embCAA58699.1	<i>N. tabacum</i>	592	2.00E-35	-1.87 (± 0.78)
GR979414	Methionine synthase	AAF74983.1	<i>S. tuberosum</i>	430	8.00E-57	1.56 (± 0.84)
GR979438	Putative S-adenosylmethionine synthetase	ACF17673.1	<i>C. annuum</i>	315	3.00E-09	-1.53 (± 0.13)
GR979436	Ribulose biphosphate carboxylase, small subunit	AAA34192.1	<i>S. lycopersicum</i>	226	6.00E-24	2.57 (± 0.67)
GR979416	S-Adenosyl-L-homocysteine hydrolase	AAV31754.1	<i>N. tabacum</i>	735	9.00E-133	2.98 (± 0.89)
GR979397	Ribulose biphosphate carboxylase/oxygenase activase	spO49074.1	<i>Lycopersicon pennellii</i>	287	3.00E-23	-1.17 (± 0.79)

Table 2 Continued.

GENBANK accession ID	Similarity of transcripts	GENBANK match	Organism	Obtained sequence length (bp)†	E-value	Fold induction (± SD)*
CELLULAR TRANSPORT						
GR979488	ABC1 family protein	refNP_565214.1	<i>A. thaliana</i>	305	3.00E-06	2.55 (± 0.54)
GR979395	Similar to aquaporin	dbjBAD95790.1	<i>S. lycopersicum</i>	488	3.00E-24	2.78 (± 0.78)
GR979445	ATP-dependent transporter, putative	EEF38635.1	<i>R. communis</i>	761	2.00E-61	2.9 (± 0.12)
GR979454	Nonspecific lipid transfer protein	embCAJ19705.1	<i>S. lycopersicum</i>	524	4.00E-59	3.71 (± 0.44)
GR979462	Vacuolar ATP synthase proteolipid subunit	EEF48309.1	<i>R. communis</i>	214	3.00E-12	1.51 (± 0.56)
GR979437	ATP synthase (γ subunit)	spP29790.1	<i>N. tabacum</i>	622	3.00E-62	2.32 (± 0.09)
GR979455	Water channel protein	dbjBAA20075.1	<i>Nicotiana excelsior</i>	591	9.00E-99	2.87 (± 0.23)
TRANSCRIPTION/TRANSLATIONAL FACTOR						
GR979443	MRNA-binding protein precursor	AAD21574.3	<i>S. lycopersicum</i>	423	2.00E-75	2.38 (± 0.13)
GR979473	Similar to elongation factor 2	ACG42954.1	<i>V. vinifera</i>	763	1.00E-116	2.62 (± 0.12)
GR979479	Eukaryotic translation initiation factor 2	ABB86256.1	<i>S. tuberosum</i>	307	4.00E-130	1.34 (± 0.59)
GR979480	Elongation factor 1-α	AAR83865.1	<i>C. annuum</i>	837	1.00E-17	2.69 (± 0.55)
GR979441	AGO4-2	EEF61505.1	<i>N. benthamiana</i>	562	6.00E-94	2.03 (± 0.67)
GR979390	Basic helix-loop-helix (bHLH) family protein†	refNP_001119080.1	<i>A. thaliana</i>	359	9.00E-22	4.63 (± 0.89)
PROTEIN DEGRADATION						
GR979393	26S proteasome AAA-ATPase subunit RPT4a†	dbjBAC23035.1	<i>S. tuberosum</i>	637	3.00E-100	4.22 (± 0.13)
GR979460	RUB1 (RELATED TO UBIQUITIN 1)	refNP_564379.2	<i>A. thaliana</i>	619	1.00E-57	3.23 (± 0.77)
GR979415	Ubiquitin-conjugating enzyme E2†	refNP_564379.2	<i>S. lycopersicum</i>	357	7.00E-54	5.55 (± 0.64)
GR979475	Armaddillo repeat motif-containing protein†	AAK60564.1	<i>N. tabacum</i>	820	5.00E-79	5.20 (± 0.98)
GR979474	Ubiquitin-associated uba/ubx domain-containing protein	EEF34340.1	<i>R. communis</i>	681	8.00E-26	3.29 (± 0.75)
GR979413	Cysteine proteinase 3	spQ40143	<i>S. lycopersicum</i>	555	2.00E-31	-2.57 (± 0.33)
DEFENCE RESPONSE						
GR979439	Cysteine protease TDI-65†	spQ40143.1	<i>S. lycopersicum</i>	682	3.00E-80	4.55 (± 0.83)
GR979391	Pathogenesis-related leaf protein 6†	spP04284.2	<i>S. lycopersicum</i>	738	3.00E-92	5.66 (± 0.41)
GR979470	Phosphoric diester hydrolase, putative	EEF40839.1	<i>R. communis</i>	341	2.00E-36	1.36 (± 0.63)
GR979458	PR8/chloroplast thiazole biosynthetic protein	AAZ93636.1	<i>N. tabacum</i>	485	2.00E-23	-1.78 (± 0.64)
PHOTOSYNTHESIS/ENERGY						
GR979428	Ferredoxin-1	AAA34192.1	<i>S. lycopersicum</i>	571	5.00E-62	2.97 (± 0.70)
GR979394	Cytochrome b6-f complex iron-sulphur subunit	spQ69GY7.1	<i>S. tuberosum</i>	404	5.00E-67	-1.13 (± 0.31)
GR979464	Thioredoxin m(mitochondrial)-type	EEF42142.1	<i>R. communis</i>	328	9.00E-41	-2.32 (± 0.63)
GR979442	NADH-ubiquinone oxidoreductase subunit-like	ABB02646.1	<i>S. tuberosum</i>	343	2.00E-59	3.79 (± 0.89)
GR979406	Oxygen-evolving enhancer protein 1	spP23322.2	<i>S. lycopersicum</i>	736	4.00E-109	-1.85 (± 0.03)
GR979423	Chlorophyll <i>a-b</i> -binding protein 4	spP14278.1	<i>S. lycopersicum</i>	553	9.00E-103	-1.88 (± 0.61)
GR979422	Chlorophyll <i>a-b</i> -binding protein	spP13869.1	<i>P. hybrida</i>	487	1.00E-62	3.39 (± 0.24)
GR979426	Photosystem I subunit XI	AAO85557.1	<i>Nicotiana attenuata</i>	271	1.00E-33	2.98 (± 0.19)
GR979398	Chlorophyll-binding protein 1B	prf1204205B	<i>S. lycopersicum</i>	694	2.00E-82	2.89 (± 0.37)
GR979432	Photosystem II 10 kDa polypeptide	spQ40163.1	<i>S. lycopersicum</i>	218	3.00E-30	2.83 (± 0.57)
GR979433	Photosystem II oxygen-evolving complex protein 3	AAU03361.1	<i>S. lycopersicum</i>	544	7.00E-75	2.07 (± 0.13)
GR979451	Chlorophyll <i>a-b</i> -binding protein 3C-like	ABB55370.1	<i>S. tuberosum</i>	744	4.00E-118	3.31 (± 0.99)
GR979402	Chlorophyll <i>a-b</i> -binding protein 5	spP14279	<i>S. lycopersicum</i>	612	1.00E-117	1.57 (± 0.17)
GR979491	Chloroplast pigment-binding protein CP29	ABG73415.1	<i>N. tabacum</i>	583	4.00E-93	2.40 (± 0.77)
GR979465	Lhca5 protein	ABN10530.1	<i>Gentiana lutea</i>	272	1.00E-06	-2.06 (± 0.21)
OTHERS/UNKNOWN FUNCTION						
GR979446	Hypothetical protein	ABK96267.1	<i>P. trichocarpa</i>	586	1.00E-87	1.48 (± 0.14)
GR979411	Unknown	ABB16969.1	<i>S. tuberosum</i>	306	1.00E-36	1.89 (± 0.22)
GR979419	Hypothetical protein	refXP_002274904.1	<i>V. vinifera</i>	691	5.00E-64	2.22 (± 1.11)
GR979418	Conserved hypothetical protein	EEF30430.1	<i>R. communis</i>	350	3.00E-05	-2.02 (± 0.97)
GR979404	Hypothetical protein LOC100193383	refNP_001131619.1	<i>Z. mays</i>	240	6.00E-29	1.08 (± 0.03)
GR979407	Unknown	ACJ84739.1	<i>Medicago truncatula</i>	518	1.00E-44	1.37 (± 0.81)
GR979408	Unknown protein	refNP_566336.1	<i>A. thaliana</i>	835	1.00E-71	1.90 (± 0.60)
GR979486	Hypothetical protein Osj_18110	EEE63300.1	<i>O. sativa</i>	536	9.00E-06	-1.98 (± 0.73)
GR979490	Predicted protein	refXP_002298321.1	<i>P. trichocarpa</i>	190	1.00E-12	1.26 (± 0.93)
GR979400	Unknown	ABB16993.1	<i>S. tuberosum</i>	570	5.00E-57	-2.00 (± 0.13)

*Fold inductions are presented as the expression ratio ToLCNDV stressed to mock of each transcript to that of tubulin with SDs. Negative values of fold induction show the down-regulation of transcripts. Standard deviations (± SD) were calculated from three independent experiments. The transcripts are listed according to their possible functions. The E-value is used to indicate the significance of sequence similarity.

†Transcripts selected for quantitative real-time polymerase chain reaction (qRT-PCR).

‡Obtained length of transcript in base pairs (bp).

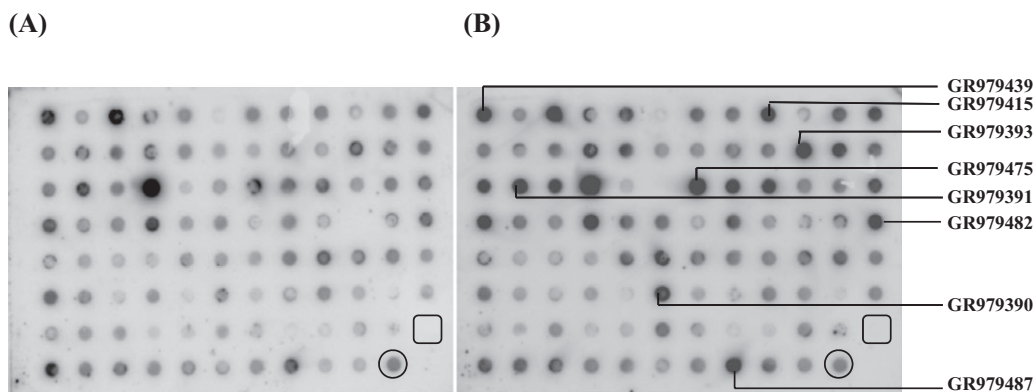


Fig. 4 Reverse Northern blot of transcripts obtained from subtracted cDNA library. DNA array hybridization was performed on a nylon membrane. Segments of representative identical nylon membranes containing cDNA spots from the subtracted cDNA library generated with *Tomato leaf curl New Delhi virus* (ToLCNDV)-infected tolerant cultivar H-88-78-1. The figure shows hybridization with ^{32}P -labelled cDNA probes prepared from equal amounts of poly(A⁺) RNA of control (A) and virus-infected (B) H-88-78-1. Circles indicate α -tubulin and squares indicate neomycin phosphotransferase II (NPTII) spots.

response, transcription process, proteolysis and hormone signalling.

qRT-PCR of the identified differentially expressed clones

To validate the changes in mRNA abundance, as detected by reverse Northern analysis, qRT-PCR was performed to evaluate quantitatively the relative abundance of eight transcripts in both H-88-78-1 and Punjab Chhuhara. All of these transcripts exhibited strong induction at 14 and 21 dpi in the tolerant cultivar in response to ToLCNDV infection. It was interesting to note that the accumulation of different transcripts occurred with different kinetics. Several transcripts were induced after 7 dpi, whereas the expression of some had declined by 21 dpi. Transcripts encoding the serine/threonine kinase PBS1 (*GR979482*), cysteine protease TDI-65 (*GR979439*), basic helix–loop–helix (bHLH) family protein (*GR979390*) and 26S proteasome AAA-ATPase subunit RPT4a (*GR979393*) were highly up-regulated at 21 dpi, whereas the ubiquitin-conjugating enzyme E2 (*GR979415*) and Armadillo repeat motif (ARM)-containing proteins (*GR979475*) showed a late response, with induction at 28 dpi in the tolerant cultivar (Fig. 5A–H).

Transcript *GR979390* encodes a bHLH transcription factor. The expression level of this transcript in the susceptible cultivar was normal, whereas, in the tolerant cultivar, the relative expression level was 3.5-fold at 7 dpi and remained constant until 21 dpi, suggesting that its induction might suppress the susceptibility to ToLCNDV infection (Fig. 5A). It is relevant to note that *Arabidopsis* bHLH (*AtMYC2*) functions as a transcriptional activator in abscisic acid (ABA) signalling (Abe *et al.*, 2003). ABA regulates processes related to abiotic and biotic stress tolerance and disease resistance (Finkelstein *et al.*, 2002; Ton *et al.*, 2009).

Although ABA has emerged as an important regulator of biotic defence responses, its role against viruses is yet to be resolved fully. ABA promotes resistance in some plant–pathogen interactions, whereas it increases susceptibility in *Arabidopsis*, soybean, potato, etc. (Asselbergh *et al.*, 2008). An elevated level of ABA in tobacco was positively correlated with increased disease resistance (Fraser, 1982; Whenham *et al.*, 1986). The increased level of this transcription factor may lead to the activation of ABA signalling, which, in turn, may regulate plant defence for enhanced tolerance to ToLCNDV.

Transcript *GR979393* showed similarity with the putative 26S proteasome AAA-ATPase subunit RPT4a of *Solanum tuberosum*. Putative 26S proteasome AAA-ATPase subunit RPT4a is a base subcomplex of the proteasome regulatory particle. This subunit uses ATP hydrolysis to assist during the recognition and/or hydrolysis of substrate by 26S proteasome (Groll and Huber, 2003; Vierstra *et al.*, 1999). Differential expression of the 26S proteasome subunit increased substantially until 21 dpi in the tolerant cultivar H-88-78-1. There was more than 2.5-fold induction in the tolerant cultivar H-88-78-1 in comparison with the mock-inoculated control. In the susceptible cultivar, it tended to increase only up to 1.7-fold (Fig. 5B). Previously, it has been suggested that the polyubiquitination of the movement protein (MP) and subsequent degradation by the 26S proteasome may play a substantial role in the regulation of virus spread (Reichel and Beachy, 2000). Intriguingly, proteasomes purified from sunflower also possess endonuclease activity capable of cleaving *Tobacco mosaic virus* (TMV) RNA (Ballut *et al.*, 2003).

Transcript *GR979415* was homologous to the ubiquitin-conjugating enzyme E2 of *Solanum lycopersicum*. This enzyme interacts with ubiquitin ligase (E3), a catalyst that mediates the final transfer of ubiquitin to the ϵ -amino group of the target protein. Hence, it is an important component of the

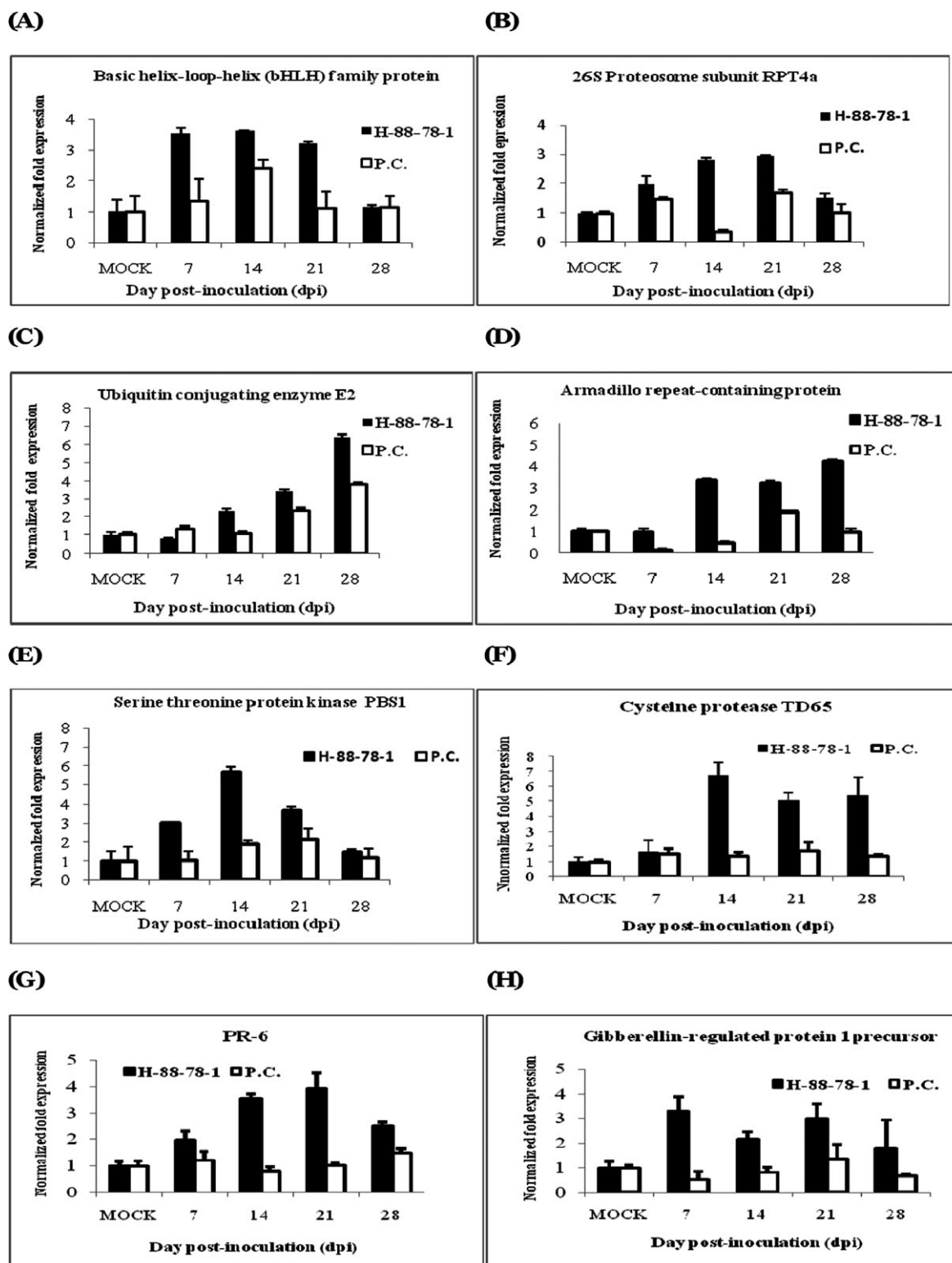


Fig. 5 Quantitative real-time polymerase chain reaction (qRT-PCR) analyses of the expression of selected transcripts in response to *Tomato leaf curl New Delhi virus* (ToLCNDV) infection in tolerant (H-88-78-1) and susceptible (Punjab Chuhhara) cultivars. Expression patterns of basic helix-loop-helix (bHLH) (A), 26S proteasome AAA-ATPase subunit RPT4a (B), ubiquitin-conjugating enzyme E2 (C), Armadillo repeat motif-containing protein (D), serine/threonine protein kinase (E), cysteine protease (F), pathogenesis-related leaf protein 6 (G) and gibberellin-regulated protein 1 (H). Bars indicate the standard deviations (\pm SD).

ubiquitin-mediated proteolysis pathway. Enhanced expression (approximately two fold) of this transcript was detected at 14 dpi in the tolerant relative to the susceptible cultivar, which steadily increased until 28 dpi (Fig. 5C). The enhanced level of the ubiquitin-conjugating enzyme E2 may be responsible for the increased processing of ubiquitin-mediated proteolysis of viral proteins. The importance of ubiquitination in plant defence has also been documented in other recent findings (Craig *et al.*, 2009; Devoto *et al.*, 2003; Glickman and Ciechanover, 2002).

Transcript *GR979475* corresponds to the ARM-containing protein of *Nicotiana tabacum*. ARM is approximately 40 amino acids in length, is typically present in a variable number of tandem copies and functions in protein–protein interactions (Hammond-Kosack and Jones, 1996; Hatzfeld, 1999). In our experiment, the basal expression of this transcript was more than three fold greater in the tolerant cultivar at 14 dpi and reached a maximum at 28 dpi (4.25-fold), whereas, in the susceptible cultivar, its expression declined to less than that of the mock-inoculated control (Fig. 5D). This suggests that ARM-containing proteins may play a unique role in the ubiquitin-mediated pathway of protein degradation in the tolerant cultivar H-88-78-1. Recently, the role of the ARM-containing U-box ligase in plant defence has been described (Dreher and Callis, 2007). In these proteins, the U-box functions as the E2 interaction domain, whereas the ARM domain might contribute directly or indirectly to substrate recognition. Several other ARM-containing proteins are induced by elicitors in several plant species and appear to participate in the plant defence response in both basal and resistance gene-mediated pathways (Dreher and Callis, 2007).

Transcript *GR979482* showed similarity with the serine/threonine protein kinase PSB1 of *Ricinus communis*. Protein kinases play a central role in signalling during pathogen recognition and the subsequent activation of the plant defence mechanism (Romeis, 2001; Swiderski and Innes 2001). This transcript was induced at all time points in the tolerant cultivar, with maximum mRNA accumulation at 14 dpi (greater than five fold), whereas, in the susceptible cultivar, its expression was lower (Fig. 5E). Some recent studies have indicated the involvement of the serine/threonine protein kinase OX11 during downstream signalling after the oxidative burst following pathogen attack (Legaz *et al.*, 1998; Rentel *et al.*, 2004). An increased level of this transcript has suggested that, possibly, the serine/threonine kinase plays an important role in ToLCNDV tolerance.

Transcript *GR979439* corresponds to the cysteine protease TDI-65 of *Solanum lycopersicum*. Recently, proteolytic enzymes and processes have been implicated or shown to play a regulatory role in the plant defence response (Bernoux *et al.*, 2008; Krüger *et al.*, 2002; Lam and Del Pozo, 2000; Nishimura and Somerville, 2002). The protease that cleaves its targets in a caspase-like manner (such as cysteine proteases in animal systems) has been found recently to be active early in the

Nicotiana–TMV interaction (Chichkova *et al.*, 2004). The expression of this transcript reached six fold at 14 dpi, followed by more than four fold induction at 21 and 28 dpi. The susceptible cultivar failed to up-regulate its expression during all time points of infection (Fig. 5F). This elevation in the level of transcript expression suggests that, in the tolerant cultivar, the early defence response, generated by the cysteine protease, is capable of suppressing the viral infection.

Transcript *GR979391* encodes pathogenesis-related leaf protein 6 (PR-6). A cysteine proteinase inhibitor class of PR-6 proteins is induced by pathogen attack (Vidyasekaran, 2002). PR-6 exhibited a rapid up-regulation as early as 7 dpi (two fold) and reached a maximum level at 21 dpi (3.9-fold) in comparison with the mock-inoculated control in the tolerant cultivar (Fig. 5G). Some PR proteins release elicitor molecules from the host cell wall surface, which may stimulate the biosynthesis of phenolic compounds, such as phytoalexins (Vidyasekaran, 2002). Plants possess both preformed and inducible mechanisms to resist pathogen invasion. The significance of PR proteins in the inhibition of pathogen infection has been reviewed in a recent article, where they were shown to induce the defence response to suppress pathogenesis (Van Loon *et al.*, 2006).

The transcript *GR979487* encodes gibberellin regulatory protein 1, which is a hormone-regulated protein which may function as a defence gene. Gibberellic acid (GA) is a growth-promoting hormone that positively regulates processes such as normal plant growth and development (Swain and Singh, 2005). The gibberellin regulatory protein transcript showed up to 3.5-fold induction at early stages of ToLCNDV infection (7 dpi) in the tolerant cultivar in comparison with the mock-inoculated control (Fig. 5H). An antimicrobial peptide Snakin-2, resembling gibberellin regulatory protein 1, was induced locally by wounding and responded to pathogen infection in a GA-regulated manner in potato (Herzog *et al.*, 1995; Lobo *et al.*, 2002). Hence, it is suggested that gibberellin regulatory protein 1 of cultivar H-88-78-1 may provide tolerance by assisting the plant to overcome ToLCNDV infection to yield normal growth and development. ToLCNDV infection in the susceptible tomato cultivar may have affected GA biosynthesis; as a result, plant growth was affected.

In summary, gene expression profiles were investigated in one tolerant and one susceptible cultivar following inoculation with ToLCNDV. In both cases, differences were found in symptom severity and in the accumulation of the viral genomic DNA component and corresponding virus-specific siRNAs at different time points (7, 14 and 21 dpi). Our limited knowledge of host gene expression during geminivirus infection *in planta* is a result of the absence of a well-characterized, compatible, virus–host system suitable for transcriptome profiling studies. Our study reveals that the changes in host gene expression that occur during ToLCNDV interaction are associated with the tolerant characteristics of cultivar H-88-78-1. A strong correlation of

siRNA accumulation with ToLCNDV tolerance was observed in cultivar H-88-78-1. A recent study has indicated the presence of a strong RNAi pathway in a soybean cultivar conferring immunity against *Mungbean yellow mosaic India virus* (Yadav *et al.*, 2009). Plants have evolved two antiviral mechanisms based on RNA degradation: RNAi- and 20S-mediated degradation (Ballut *et al.*, 2005). 20S-mediated RNA degradation can be considered as the first component of the plant antiviral defence, targeting nonhost RNAs, a less fine-tuned mechanism than RNAi (Dielens *et al.*, 2009). In the past few years, the ubiquitin proteasomal system (UPS) has emerged as an essential protagonist in plant–pathogen interactions. Viral proteins themselves are the target of UPS (Reichel and Beachy, 2000). In our study, most of the up-regulated transcripts (GR979393, GR979460, GR979415, GR979475 and GR979474) identified were involved in UPS. The induction of these transcripts at different stages of ToLCNDV infection suggests that they function in providing tolerance to ToLCNDV infection in the tolerant cultivar H-88-78-1. It should be highlighted that, among the various components of protein degradation, defence signalling transcripts are over-expressed in response to pathogen attack. Over-expression of transcripts, such as bHLH transcription factors (GR979390) and gibberellin-regulated protein 1 (GR979487), during ToLCNDV infection advocates coordination among hormone signalling and defence response pathways (Asselbergh *et al.*, 2008; Herzog *et al.*, 1995; Lobo *et al.*, 2002). Further, characterization and functional analysis of these identified genes can lead to a better understanding of host defence during interaction with begomoviruses. With the ongoing effort to sequence the tomato genome, the identified transcripts may play an important role in breeding for tomato improvement and the development of cultivars with improved disease resistance.

EXPERIMENTAL PROCEDURES

Construction of infectious ToLCNDV clone

Tandem repeat constructs of ToLCNDV genomic components (DNA-A and DNA-B) were available from the Molecular Virology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi, India (Chakraborty *et al.*, 2008). These constructs were re-cloned into pCambia2301 at the *Sma*I site and confirmed by restriction digestion using appropriate enzymes.

Agroinoculation

Tandem repeat constructs of DNA-A and DNA-B of ToLCNDV were introduced into *Agrobacterium tumefaciens* strain EHA105 by the freeze–thaw method. *Agrobacterium tumefaciens* harbouring DNA-A and DNA-B was grown overnight in yeast extract broth (YEB) (pH 7.0) at 28 °C. The cultures were centrifuged at

5000 g for 10 min and the cell pellets were suspended in YEB (pH 5.6) with 100 mM acetosyringone (Sigma, USA). Tomato seeds were germinated in vermiculite to the two-leaf stage. Agroinoculation was performed by the stem inoculation method, in which wounds were made around the growing nodal region of the tomato stem by pricking three to four times with a 30-gauge needle. About 20 µL of *Agrobacterium* suspension containing an equimolar concentration of *A. tumefaciens* harbouring DNA-A and DNA-B were mixed and applied to the wounds. For mock inoculation, *Agrobacterium* harbouring pCambia2301 alone was used. The plants were kept in a glasshouse at 25 °C for symptom development until 45 dpi.

Southern hybridization

The leaf samples (inoculated as well as systemic) from ToLCNDV-infected and mock-inoculated plants were collected at 7, 14 and 21 dpi. Total DNA from symptomatic leaves of tomato cultivars (five plants per treatment) were isolated by the cetyltrimethylammonium bromide (CTAB) method (Porebski *et al.*, 1997). For Southern hybridization, 5 µg of total DNA was electrophoresed on 1% agarose gel in TBE [Tris-borate EDTA; 45 mM Tris-borate, 1 mM EDTA (pH 8)]. Samples were transferred to hybrid-N⁺ membrane (Amersham Bioscience, USA) and hybridized with a α^{32} P-deoxycytidine triphosphate (dCTP)-labelled DNA-A-specific coat protein (CP) sequence amplified using 5'-ACAGAAAACCCAGAATGTACAGAA-3' and 5'-CAACATTAA GGCATTTTCAGTATG-3'. Radiolabelled probe was prepared and hybridized by a high prime DNA labelling kit (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol.

Detection of virus-specific siRNA

Total RNA from infected as well as mock-inoculated tomato leaves was isolated using TRIzol reagent (Sigma). Total RNA (50 µg) was resuspended in formamide RNA loading dye and denatured at 95 °C for 2 min. Samples were electrophoresed on 15% polyacrylamide (19 : 1) gel containing 7 M urea in 0.5 × TBE, and transferred to nylon membrane according to the procedure described by Sambrook and Russell (2001). The Rep gene of ToLCNDV was PCR amplified using specific primer pairs (5'-CTCAAAGGTGTATAGCAATGATGC-3' and 5'-AAAGTCGAAT CTGTTATTT-3') for probe preparation. Probes were labelled with α^{32} P-dCTP using a high prime DNA labelling kit (Roche). Hybridization with radiolabelled probe was performed overnight at 42 °C. The blot was scanned in a Phosphor-imager (Typhoon-9210, GE Healthcare, NJ, USA) and quantified using Bio-Rad Quantity One software (USA).

Construction of subtracted cDNA library

Frozen leaves were ground in liquid nitrogen and total RNA was isolated using TRIzol reagent (Sigma) according to the manufac-

turer's instructions. RNA integrity was examined by electrophoresing samples on denaturing formaldehyde–1.2% agarose–ethyl bromide gel. The quantity and quality of isolated total RNA was examined spectrophotometrically. Messenger RNA (mRNA) was purified from total RNA using the MagneSphere mRNA Purification Kit (Promega, Madison, USA) according to the manufacturer's protocol. A forward subtraction was performed between 21-day-old ToLCNDV-infected H-88-78-1 as tester and mock-infected H-88-78-1 (with *Agrobacterium* harbouring vector pCAMBIA2301 only) as driver. Purified mRNA (2 µg) was used for reverse transcription and first-strand cDNA thus prepared was used for SSH with the Clontech PCR Select-cDNA Subtraction kit (BD Biosciences, Clontech, CA, USA). In brief, driver and tester cDNAs were *RsaI* digested, extracted with phenol–chloroform, ethanol precipitated and resuspended in water. Tester cDNA was split into two pools and each was ligated to a different adapter (provided with the cDNA subtraction kit). Unsubtracted tester control cDNA was ligated to both adapters. Two rounds of hybridization and PCR amplification were carried out to normalize and enrich the differentially expressed cDNAs according to the manufacturer's protocol, with the following changes: the primary PCR was performed for 30 cycles (94 °C, 30 s; 65 °C, 30 s; 72 °C, 90 s) and the secondary PCR was performed for 16 cycles (94 °C, 30 s; 66 °C, 30 s; 72 °C, 90 s). Products of the secondary PCR were purified and cloned into pGEMT-easy vector (Promega) and transformed into DH5α *Escherichia coli* competent cells.

DNA sequencing and data analysis

Sequences of the recombinant plasmids were determined with an automated sequencer (ABI Sequencer, Version No.3770, Applied Biosystems, USA) using M13 forward and reverse primers. Nucleotides and translated sequences were compared with nonredundant sequences of the GENBANK database using the BLAST sequence alignment program (Altschul *et al.*, 1997). ESTs of more than 100 nucleotides in length and an E-value of less than 1E-05 were considered to be significant. Further functional classification was performed according to the MIPS data base (http://mips.helmholtz-muenchen.de/proj/funecatDB/search_main_frame.html).

Reverse Northern hybridization

Individual clones of the subtracted cDNA library were amplified in a 96-well PCR plate using M13 forward and reverse primers in a 50-µL reaction at an annealing temperature of 60 °C for 30 cycles. The products were analysed on agarose gel to confirm the insert size, quality and quantity. Purified PCR products were denatured by adding an equal volume of 0.6 M sodium hydroxide. An equal volume of each denatured PCR product (about

100 ng) of above 300 bp in size was spotted onto two Hybond N⁺ membranes (Amersham Bioscience) using a BIO-DOT dot-blot apparatus (Bio-Rad) in 96-well formats to prepare two identical arrays. In addition, a PCR product of α-tubulin cDNA (produced using primer sequences 5'-GGTACTGGTCTTCAAGGTTTC-3' and 5'-TTGTCATAGATAGCCTCATTGT-3') was spotted as internal control to normalize the signals of two different blots corresponding to ToLCNDV-treated and mock-treated samples. A PCR product of the neomycin phosphotransferase II (NPTII) gene from the vector pCAMBIA 1305.1 using primer sequences (5'-TTTTCTCCAATCAGGCTTG-3' and 5'-TCAGGCTCTTCACTCCATC-3') was also spotted as a negative control to subtract the background noise. The membranes were neutralized with neutralization buffer (0.5 M Tris-Cl, pH 7.4, 1.5 M NaCl) for 3 min, washed with 2% standard saline citrate (SSC) and cross-linked using UV cross-linker (Stratagene, La Jolla, CA, USA).

qRT-PCR analysis

For eight highly up-regulated transcripts, the differential expression was confirmed using qRT-PCR. Total RNA was isolated at 7, 14, 21 and 28 dpi from the leaves of ToLCNDV- and mock-inoculated tolerant (H-88-78-1) and susceptible (Punjab Chuhara) tomato cultivars by TRIzol reagent (Sigma). Total RNA (2 µg) was used to synthesize first-strand cDNA from each sample using Superscript reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the supplier's manual. The primers used for qRT-PCR were designed from the sequences of selected transcripts using Primer Express Version 3.0 (Table 3). qRT-PCR was performed on a Step One Real-Time PCR System (Applied Biosystems) using Power SYBR Green dye (Applied Biosystems). PCR was performed for each sample in triplicate; α-tubulin, a constitutively expressed protein, was used as internal control. The amount of transcript of each gene, normalized to the internal control α-tubulin, was analysed using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). The amount of transcript of each target gene under the mock condition was designated as 1.0. The PCR conditions were kept as 95 °C for 10 min, 95 °C for 15 s, 60 °C for 1 min for 40 cycles, 95 °C for 15 s and 60 °C for 1 min. The experiment was repeated three times to check the reproducibility.

ACKNOWLEDGEMENTS

We are grateful to the Director, National Institute of Plant Genome Research (NIPGR) for providing facilities, to the Council for Scientific and Industrial Research, Government of India for providing a Senior Research Fellowship to Mr Neeraj K Rai, and to the Department of Biotechnology, Government of India for providing financial support (Grant no. BT/PR/5274/AGR/16/464/2004). We thank Mr Rajeev K. Yadav, Ms Swati Puranik and Ms Kajal Kumari (NIPGR, New Delhi, India) for help with the

Table 3 Primers used for quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

Primer ID	Amplicon length (bp)	Forward sequence (5'–3')	Reverse sequence (5'–3')
26S proteasome AAA-ATPase subunit RPT4a	61	GGGCTGATATGCGGAATGTT	TCACGCTCTGCACGAATTG
Armadillo repeat motif-containing protein	65	GCGATCTCTGCACGCAAA	TGGATAGCTCCAAAAGCAGATG
Basic helix–loop–helix protein	66	CACCGGATGGGTCAACATC	TCTTCTCTATTCTCTCTGATACAA
Cysteine protease	56	TCCGAAGGCCCAATAGG	CACTGGGAGTGAAGGCAATGA
Gibberellin-regulated protein 1	63	CATTTTCATCCTCCTACACAGTT	TTGCTGCCTCTATCTGTTCAGT
Pathogenesis-related leaf protein 6	61	AGGGCAGCCGTGCAATT	ATTGGCTGGTAGCGTGGTTATAG
Serine/threonine protein kinase	63	TGCATTGCAAACAGCAACAA	CCAAGAGATCCTTACCAATGAG
α-Tubulin	62	AACCTGTGAGATAAGCGGATTGA	GGCGCTCATTGGACATTGA
Ubiquitin-conjugating enzyme E2	60	TCAGCTGGACCCATGATTGT	TGCTGGCCAGTAGCTGAA

manuscript. We would like to thank the reviewers for their critical comments.

REFERENCES

- Abe, H., Urao, T., Ito, T., Seki, M., Shinozaki, K. and Shinozaki, K.Y. (2003) *Arabidopsis* AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. *Plant Cell*, **15**, 63–78.
- Akbergenov, R., Si-Ammour, A., Blevins, T., Amin, I., Kutter, C., Vanderschuren, H., Zhang, P., Gruissem, W., Meins, Jr., F., Hohn, T. and Pooggin, M.M. (2006) Molecular characterization of geminivirus-derived small RNAs in different plant species. *Nucleic Acids Res.* **34**, 462–471.
- Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402.
- Asselbergh, B., Vleeschauwer, D.D. and Hofte, M. (2008) Global switches and fine-tuning—ABA modulates plant pathogen defense. *Mol. Plant–Microbe Interact.* **21**, 709–719.
- Ballut, L., Petit, F., Mouzeyar, S., Le Gall, O., Candresse, T., Schmid, P., Nicolas, P. and Badaoui, S. (2003) Biochemical identification of proteasome-associated endonuclease activity in sunflower. *Biochim. Biophys. Acta*, **1645**, 30–39.
- Ballut, L., Drucker, M., Pugniere, M., Cambon, F., Blanc, S., Roquet, F., Candresse, T., Schmid, H.-P., Nicolas, P., Gall, O.L. and Badaoui, S. (2005) HcPro, a multifunctional protein encoded by a plant RNA virus, targets the 20S proteasome and affects its enzymic activities. *J. Gen. Virol.* **86**, 2595–2603.
- Bernoux, M., Timmers, T., Jauneau, A., Briere, C., de Wit, P.J.G.M., Marco, Y. and Deslandes, L. (2008) RD19, an *Arabidopsis* cysteine protease required for RRS1-R-mediated resistance, is relocalized to the nucleus by the *Ralstonia solanacearum* PopP2 effector. *Plant Cell*, **20**, 2252–2264.
- Chakraborty, S. (2008) *Tomato leaf curl viruses* from India (*Geminiviridae*). In: *Encyclopedia of Virology* (Mahy, B.W.J. and Van Regenmortel, M.H.V., eds), pp. 124–133. Oxford: Elsevier.
- Chakraborty, S., Vanitharani, R., Chattopadhyay, B. and Fauquet, C.M. (2008) Supervirulent pseudorecombination and asymmetric synergism between genomic components of two distinct species of begomovirus associated with severe tomato leaf curl disease in India. *J. Gen. Virol.* **89**, 818–828.
- Chellappan, P., Vanitharani, R., Pita, J. and Fauquet, C.M. (2004) Short interfering RNA accumulation correlates with host recovery in DNA virus-infected hosts, and gene silencing targets specific viral sequences. *J. Virol.* **78**, 7465–7477.
- Chichkova, N.V., Kim, S.H., Titova, E.S., Kalkum, M., Morozov, V.S., Rubtsov, Y.P., Kalinina, N.O., Taliany, M.E. and Vartapetian, A.B. (2004) A plant caspase like protease activated during the hypersensitive response. *Plant Cell*, **16**, 157–171.
- Collazo, C., Romos, P.L., Chacon, O., Borroto, C.J., Lopez, Y., Pozol, M., Thomma, B.H.P., Hein, I. and Borrás-Hidalgo, O. (2005) Phenotypical and molecular characterization of the *Tomato mottle taino virus–Nicotiana megalosiphon* interaction. *Physiol. Mol. Plant Pathol.* **67**, 231–236.
- Craig, A., Ewan, R., Mesmar, J., Gudipati, V. and Sadanandom, A. (2009) E3 ubiquitin ligases and plant innate immunity. *J. Exp. Bot.* **60**, 1123–1132.
- Czosnek, H. and Laterrot, H. (1997) A worldwide survey of *Tomato yellow leaf curl viruses*. *Arch. Virol.* **142**, 1391–1406.
- Devoto, A., Muskett, P.R. and Shirasu, K. (2003) Role of ubiquitination in the regulation of plant defense against pathogens. *Curr. Opin. Plant Biol.* **6**, 307–311.
- Dielen, A.S., Badaoui, S., Candresse, T. and German-Retana, S. (2009) The ubiquitin/26S proteasome system in plant–pathogen interactions: a never-ending hide-and-seek game. *Mol. Plant Pathol.* **11**, 293–308.
- Ding, S.W., Li, H., Lu, R., Li, F. and Li, W.X. (2004) RNA silencing: a conserved antiviral immunity of plants and animals. *J. Virus Res.* **102**, 109–115.
- Dreher, K. and Callis, J. (2007) Ubiquitin, hormones and biotic Stress. *Ann. Bot.* **99**, 787–822.
- Finkelstein, R.R., Gampala, S.S.L. and Rock, C.D. (2002) Abscisic acid signaling in seed and seedling. *Plant Cell*, **14**, S15–S45.
- Fraser, R.S.S. (1982) Are 'pathogenesis related' proteins involved in acquired systemic resistance of tobacco plants to *Tobacco mosaic virus*. *J. Gen. Virol.* **58**, 305–313.
- Glickman, M.H. and Ciechanover, A. (2002) The ubiquitin proteasome proteolytic pathway: destruction for the sake of construction. *Physiol. Rev.* **82**, 373–428.
- Green, S.K. and Kalloo, G. (1994) *Leaf curl and yellowing viruses* of pepper and tomato: an overview. Technical Bulletin No. 21, Asian Vegetable Research and Development Centre, Taiwan, p. 51.
- Groll, M. and Huber, R. (2003) Substrate access and processing by the 20S proteasome core particle. *Int. J. Biochem. Cell Biol.* **35**, 606–616.

- Gutierrez, C. (1999) Geminivirus DNA replication. *Cell. Mol. Life Sci.* **56**, 313–329.
- Hammond-Kosack, K.E. and Jones, J.D.G. (1996) Resistance gene-dependent plant defense responses. *Plant Cell*, **8**, 1773–1791.
- Hammond-Kosack, K.E. and Jones, J.D.G. (2000) Responses to plant pathogens. In: *Biochemistry and Molecular Biology of Plants* (Buchanan, B., Gruissem, R. and Jones, R., eds), pp. 1102–1156. Rockville, MD: American Society of Plant Physiologists.
- Hatzfeld, M. (1999) The armadillo family of structural proteins. *Int. Rev. Cytol.* **186**, 179–224.
- Herzog, M., Dorne, A.M. and Grellet, F. (1995) GASA a gibberellin regulated gene family from *Arabidopsis thaliana* related to the tomato GAST1 gene. *Plant Mol. Biol.* **27**, 743–752.
- Jeske, H., Lutgemeier, M. and Preiss, W. (2001) DNA forms indicate rolling circle and recombination-dependent replication of *Abutilon mosaic virus*. *EMBO J.* **20**, 6158–6167.
- Kirithi, N., Maiya, S.P., Murthy, M.R.N. and Savithri, H.S. (2002) Evidence of recombination among the tomato leaf curl virus strains/species from Bangalore, India. *Arch. Virol.* **147**, 255–272.
- Krüger, J., Thomas, C.M., Golstein, C., Dixon, M.S., Smoker, M., Tang, S., Mulder, L. and Jones, J.D.G. (2002) A tomato cysteine protease required for Cf-2-dependent disease resistance and suppression of autonecrosis. *Science*, **296**, 744–747.
- Lam, E. and Del Pozo, O. (2000) Caspase-like protease involvement in the control of plant cell death. *Plant Mol. Biol.* **44**, 417–428.
- Legaz, M.E.R., de Armas, D., Pinon, C. and Vicente, C. (1998) Relationships between phenolics-conjugated polyamines and sensitivity of sugarcane to smut (*Ustilago scitaminea*). *J. Exp. Bot.* **49**, 1723–1728.
- Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^{ΔΔCt} method. *Methods*, **25**, 402–408.
- Lobo, M.B., Segura, A., Mozeno, M., Lopez, G., Olmedo, G. and Molina, A. (2002) Snakin-2, an antimicrobial peptide from potato whose gene is locally induced by wounding and responds to pathogen infection. *Plant Physiol.* **128**, 951–961.
- Lucoli, A., Noris, E., Brunetti, A., Tavazza, R., Ruzza, V., Castillo, A.G., Bejarano, R., Accotto, G.P. and Tavazza, M. (2003) *Tomato yellow leaf curl Sardinia virus* rep-derived resistance to homologous and heterologous geminiviruses occurs by different mechanisms and is overcome if virus-mediated transgene silencing is activated. *J. Virol.* **77**, 6785–6798.
- McDowell, J.M. and Dangle, J.L. (2000) Signal transduction in the plant immune response. *Trends Biochem. Sci.* **25**, 79–82.
- Moissiard, G. and Voinnet, O. (2004) Viral suppression of RNA silencing in plants. *Mol. Plant Pathol.* **5**, 71–82.
- Nawaz-ul-Rehman, M.S., Mansoor, S., Briddon, R.W. and Fauquet, C.M. (2009) Maintenance of an Old World betasatellite by a New World helper begomovirus and possible rapid adaptation of the betasatellite. *J. Virol.* **83**, 9347–9355.
- Nishimura, M. and Somerville, S. (2002) Resisting attack. *Science*, **295**, 2032–2033.
- Porebski, S., Bailey, L.G. and Baur, B.R. (1997) Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Mol. Biol. Rep.* **15**, 8–15.
- Preiss, W. and Jeske, H. (2003) Multitasking in replication is common among geminiviruses. *J. Virol.* **77**, 2972–2980.
- Reichel, C. and Beachy, R.N. (2000) Degradation of *Tobacco mosaic virus* movement protein by the 26S proteasome. *J. Virol.* **74**, 3330–3337.
- Rentel, M.C., Lecourieux, D., Ouaked, F., Usher, S.L., Petersen, L., Okamoto, H., Knight, H., Peck, S.C., Grierson, C.S., Hirt, H. and Knight, M.R. (2004) OX11 kinase is necessary for oxidative burst-mediated signalling in *Arabidopsis*. *Nature*, **427**, 858–861.
- Ribeiro, S.G., Lohuis, H., Goldbach, R. and Prins, M. (2007) *Tomato chlorotic mottle virus* is a target of RNA silencing but the presence of specific short interfering RNAs does not guarantee resistance in transgenic plants. *J. Virol.* **81**, 1563–1573.
- Rodriguez-Negrete, E.A., Carrillo-Tripp, J. and Rivera-Bustamante, R.F. (2008) RNA silencing against Geminivirus: complementary action of PTGS and TGS in host recovery. *J. Virol.* **83**, 1332–1340.
- Romeis, T. (2001) Protein kinases in the plant defense response. *Curr. Opin. Plant Biol.* **4**, 807–814.
- Ruiz-Medrano, R., Moya, J.H., Xoconostle-Cazares, B. and Lucas, W.J. (2007) Influence of cucumber mosaic virus infection on the mRNA population present in the phloem translocation stream of pumpkin plants. *Funct. Plant Biol.* **34**, 292–301.
- Saikia, A.K. and Muniyappa, V. (1989) Epidemiology and control of *Tomato leaf curl virus* in Southern India. *Trop. Agric.* **66**, 350–354.
- Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edn, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Swain, S.M. and Singh, D.P. (2005) Tale tales from sly dwarves: novel functions of gibberellins in plant development. *Trends Plant Sci.* **10**, 123–129.
- Swiderski, M.R. and Innes, R.W. (2001) The *Arabidopsis* *PBS1* resistance gene encodes a member of a novel protein kinase subfamily. *Plant J.* **26**, 101–112.
- Szitty, G., Molnar, A., Silhavy, D., Hornyik, C. and Burgyan, J. (2002) Short defective interfering RNAs of *Tombusviruses* are not targeted but trigger post-transcriptional gene silencing against their helper virus. *Plant Cell*, **14**, 359–372.
- Takemoto, D. and Hardham, A.R. (2004) The cytoskeleton as a regulator and target of biotic interactions in plants. *Plant Physiol.* **136**, 3864–3886.
- Thordal-Christian, H. (2003) Fresh insights into processes of non-host resistance. *Curr. Opin. Plant Biol.* **6**, 351–357.
- Ton, J., Victor, F. and Brigitte, M.M. (2009) The multifaceted role of ABA in disease resistance. *Trends Plant Sci.* **14**, 310–317.
- Van Loon, L.C., Rep, M. and Pieterse, C.M.J. (2006) Significance of inducible defense-related proteins in infected plants. *Annu. Rev. Phytopathol.* **44**, 135–162.
- Vanitharani, R., Chellappan, P. and Fouquet, C.M. (2003) Short interfering RNA-mediated interference of gene expression and viral DNA accumulation in cultured plant cells. *Proc. Natl. Acad. Sci. USA*, **100**, 9632–9636.
- Vanitharani, R., Chellappan, P. and Fouquet, C.M. (2005) Geminiviruses and RNA silencing. *Trends Plant Sci.* **10**, 144–151.
- Vasudeva, R.S. and Samraj, J. (1948) Leaf curl disease of tomato. *Phytopathology*, **38**, 364–369.
- Vidyasekaran, P. (2002) Bacterial disease resistance in plants. In: *Molecular Biology and Biotechnological Application* (Vidyasekaran, P., ed.), pp. 185–293. New York: The Howarth Press.
- Vierstra, R.D., Fu, H., Girod, P.A., Doelling, J.H., Nocker, S.V., Hochstrasser, M. and Finley, D. (1999) Structure and functional analysis

- sis of the 26S proteasome subunit from plants. *Mol. Biol. Rep.* **26**, 137–146.
- Voinnet, O.** (2001) RNA silencing as a plant immune system against viruses. *Trends Genet.* **17**, 449–459.
- Voinnet, O.** (2005) Induction and suppression of RNA silencing: insights from viral infections. *Nat. Rev. Genet.* **6**, 206–220.
- Waterhouse, P.M., Wang, M.B. and Lough, T.** (2001) Gene silencing as an adaptive defense against viruses. *Nature*, **411**, 834–842.
- Whenham, R.J., Fraser, R.S.S., Brown, L.P. and Payne, J.A.** (1986) *Tobacco mosaic virus* induced increase in abscisic acid concentration in tobacco leaves: intracellular location in light and dark green areas, and relationship to symptom development. *Planta*, **168**, 592–598.
- Whitham, S.A., Quan, S., Chang, H.S., Cooper, B., Estes, B., Zhu, T., Wang, X. and Yu-Ming, H.** (2003) Diverse RNA viruses elicit the expression of common sets of genes in susceptible *Arabidopsis thaliana* plants. *Plant J.* **33**, 271–283.
- Xiong, L., Lee, M.W., Qi, M. and Yang, Y.** (2001) Identification of defense-related rice genes by suppression subtractive hybridization and differential screening. *Mol. Plant–Microbe Interact.* **14**, 685–692.
- Yadav, R.K., Shukla, R.K. and Chattopadhyay, D.** (2009) Soybean cultivar resistant to Mungbean yellow mosaic India virus infection induces viral RNA degradation earlier than the susceptible cultivar. *Virus Res.* **144**, 89–95.