Dicer 1 of *Candida albicans* cleaves plant viral dsRNA in vitro and provides tolerance in plants against virus infection

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Abstract Most of the viral diseases of plants are caused by RNA viruses which drastically reduce crop yield. In order to generate resistance against RNA viruses infecting plants, we isolated the dicer 1 protein (CaDcr1), a member of RNase III family (enzyme that cleaves double stranded RNA) from an opportunistic fungus *Candida albicans*. In vitro analysis revealed that the CaDcr1 cleaved dsRNA of the coat protein gene of cucumber mosaic virus (genus *Cucumovirus*, family *Bromoviridae*). Furthermore, we developed transgenic tobacco plants (*Nicotiana tabacum* cv. Xanthi) over-expressing expressing CaDcr1 by *Agrobacterium* mediated transformation. Transgenic tobacco lines were able to suppress infection of an Indian isolate of potato virus X (genus *Potexvirus*, family *Alphaflexiviridae*). The present study demonstrates that CaDcr1 can cleave double stranded replicative intermediate and provide tolerance to plant against RNA viruses.

Keywords Dicer · Ribonuclease III · Transgenic · Fungus · RNA virus · Plants · *Candida albicans*

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Introduction

Cellular ribonucleases play central roles in cell viability owing to their involvement in maturation and degradation of various RNAs. The RNA processing events involve divergent ribonucleases including single subunit RNase A as well as highly specialized multi-subunit RNase P. The processing machinery also contains other RNA components like spliceosome [5]. RNases differ from one another in their substrate selection as well as site of cleavage. Thus, they may be considered as either exo- or endo-nucleases acting on either single or double stranded RNA. For example, endo nuclease RNase E recognizes single-stranded RNAs (ssRNA), while RNase III binds to double-stranded RNAs (dsRNA) only.

RNase III is a crucial RNA processing enzyme in bacteria as well as eukaryotes. Homologues of RNase III have also been found in worm, mouse and humans [4, 30]. On the basis of structural and evolutionary characteristics RNase III family can be categorized into two subfamilies. The bacterial RNase III subfamily is characterized by the presence of RNA binding domain (RBD) and nuclease domain [26, 27] and consists of single form. Whereas, the eukaryotic RNase III subfamily has three isoforms and is characterized by the presence of dsRBD [10, 14, 18]. All the eukaryotic RNase III differs in the number of nuclease domain and composition of N-terminal domain. Two eukaryotic RNase III have been characterized from *Saccharomyces cerevisiae* (*Rnt1*) [9] and *Schizosaccharomyces pombe* (*Pac1*) [15, 30, 37]. Similarly, an RNase III from *Candida albicans* (CaDcr1) has been isolated recently and shown to possess dicer activity such as siRNAs generation for the RNAi pathway [2]. *Candida albicans* has CaDcr1 (1836 nt), only active RNase III which has additional function of biogenesis of spliceosome and ribosome.

CaDcr1 has a single RNase III domain adjacent to the dsRBD [8]. Presence of RNase III signature motif and active-site residues required for dsRNA recognition suggests that the RNase III cleavage activity is conserved in CaDcr1 [22, 23, 36].

Viral diseases are major threats for cultivation of crop plants throughout the world as infection results in significant losses in crop yields [34]. RNA viruses are predominant in both tropical and temperate regions of the world [33]. Breeding for disease resistance seemed to be the most appropriate strategy for management of plant viruses. However, development of new variants/species due to error prone replication of RNA viruses has often led to breakdown of resistance in crop plants. This necessitated development of an alternate strategy for resistance against RNA viruses. In this study, we isolated and purified the dicer from *C. albicans* (CaDcr1) and characterized the enzymatic activity of the isolated protein. Furthermore, we developed transgenic tobacco (*Nicotiana tabacum* cv. Xanthi) expressing CaDcr1 using *Agrobacterium* mediated transformation and challenged transgenic plants with *Potato virus X* (PVX, genus *Potexvirus*; family *Alphaflexiviridae*). Our results demonstrated that purified CaDcr1 protein can cleave plant viral dsRNA in vitro and provide tolerance in tobacco.

Materials and methods

Cloning, expression and purification of CaDcr1

Total RNA from *C. albicans* strain CAI4 was isolated following standard protocol and mRNA was separated by oligo-dT method. From the mRNA, cDNA synthesis was carried out using reverse transcriptase following manufacturer's protocol (Thermo Fisher Scientific, Waltham, Massachusetts, USA). CaDcr1 was amplified from cDNA of *C. albicans* using specific primers (Forward primer 5' GGATCCATGTCATCGTTTTCTCAA 3'; Reverse primer 5' AAGCTTTTATTCTGGTGAGTCCTTAGT 3') and cloned in pTZ57R/T as per manufacturer's instructions [InsTAclone^{TM} PCR product Cloning Kit (#K1214), MBI Fermentas, India]. Subsequently, CaDcr1 ORF was cloned into the pET-28a(+) expression vector at *Bam*HI and *Hind*III sites (Promega, Madison City, USA) and the recombinant plasmid pET-CaDcr1 was confirmed by nucleotide sequencing at the DNA Sequencing facility, University of Delhi South Campus, New Delhi.

CaDcr1 protein was expressed in *Escherichia coli* strain BL21 DE3 with 0.2 mM IPTG for 3 h at 37 °C. In spite of repeated attempts, we failed to obtain the recombinant protein in the soluble fraction. Since most of the fractions formed aggregates, 8 M urea solution was used to dissolve

the inclusion bodies present in the pellet. A column packed with Ni-NTA beads (Merck, Darmstadt, Germany) was equilibrated with buffer [50 mM Tris buffer (pH 8.0), 100 mM NaCl] and protein pellet dissolved in urea was subsequently passed through it. The fractions were collected with elution buffer containing increasing amount of imidazole and considerable amount of fractions were collected at 120 mM imidazole concentration. The eluted protein was stored at – 20 °C in 50% glycerol. Further, recombinant protein was confirmed by western blot analysis using anti-His antibody and matrix-assisted laser desorption ionization (MALDI) analysis (Supplementary Fig. S1).

Cloning and in vitro transcription of the coat protein gene (CP) of CMV

The CP genes of two CMV isolates (Tfr-In and Pun-In) were amplified using specific primers (BM05F: 5'AGTCGAGTCATGGACAAATC 3' and BM06R: 5'TTAGACTGGGAGCACCC 3') and cloned in pGEM-T Easy vector in both orientations (sense i.e. Pun-In isolate and anti-sense i.e. Aur-In isolate) at *Eco*RI sites [11]. The dsRNA substrate was prepared by annealing the complementary RNA encoded by both PUNE and AUR isolates. Sense strand (691 nucleotides) was synthesized by T7 RNA polymerase-mediated transcription of *Pst*I-cleaved PUNE plasmid while the complementary (701 nucleotides) was obtained by T7 RNA polymerase-mediated transcription of *Pst*I-cleaved AUR plasmid. In vitro transcription was performed using the Riboprobe kit along with the positive control template following manufacturer's recommendations (Promega, Madison City, USA).

dsRNA degradation assay of CMV transcripts

In vitro transcribed RNAs were purified from agarose gel as described by Sambrook and Russell [31], and both the transcripts were labeled with α 32P deoxycytidine triphosphate (dCTP). Hybridization temperature of the in vitro transcripts was calculated using both GeneBee software (Supplementary Fig. S2) and Poland software (Supplementary Fig. S3). Transcripts were annealed in hybridization buffer [0.2 M NaOH (pH 6.7), 2 M NaCl, 5 mM EDTA (pH 8.0)] by heating at 85 °C for 10 min, then incubating at 45 °C for 8 h. The annealed product was precipitated using 3 M NaCl and ethanol, and dissolved in 10 μ l of DEPC treated water. An aliquot of the annealed product was subsequently added to the RNase digestion buffer [10 μ g RNase III, 50 mM tri (pH 8) and 200 mM NaCl] and incubated at 30 °C for 1 h. Further, the enzyme was denatured using sodium dodecyl sulphate (SDS) and protease K and α 32P-labeled RNA products were purified

by standard protocol [31]. Purified pellet was denatured with formaldehyde, separated on 1.2% agarose gels and the gels were exposed to the X-ray film. Radioactivity bound to the gel was detected.

Preparation of CaDcr1 construct and transformation of tobacco

The allergenicity of the CaDcr1 protein was predicted using computer aided programmes such as Food Allergy Research and Resource Program (FARRP) and Structural Database of Allergenic Proteins (SDAP). CaDcr1 was subsequently cloned into plant transformation vector, pBIN-AR at *Xba*I and *Sall* sites. *Agrobacterium tumefaciens* containing the construct, pBIN-CaDcr1 was used to transform *Nicotiana tabacum* by leaf disc method [13]. Briefly, young tobacco leaf disks (1 cm²) were immersed in *Agrobacterium* culture dissolved in Murashige and Skoog liquid medium for 10 min. The leaf explants were cultured for 2 days on medium and were subsequently transferred to the selection medium until shoot emerged (3–4 weeks). Individual shoots were then transferred to the rooting medium and the rooted plants were transferred to pots for adaptation in glass house. Seeds were collected from T₀ generation plants and grown on Kanamycin (50 mg/L) media. The Seeds of transgenic lines, which showed Mendelian pattern of inheritance, were carried over to the next generation.

The total genomic DNA was isolated from the transformed plants following standard method [6] and the presence of CaDcr1 was confirmed by PCR using gene specific primers. The seeds of *N. tabacum* lines over-expressing CaDcr1 were advanced till T₂ generation. The seedlings were transferred to soil 1 week post seed germination.

Challenge inoculation of transgenic tobacco

Six transgenic lines of *N. tabacum* plants (NT-3-1, NT-3-2, NT-5-1, NT-5-2, NT-18-1 and NT-18-2) of T₂ generation (at the five-leaf stage) were inoculated with PVX. PVX inoculum was prepared by grinding infected tobacco leaves in phosphate buffer of pH 7.0 (1.0 g/ml) and sap inoculation was carried out by rubbing the test plants predestined with carborundum powder (600 mesh). Plants rubbed with phosphate buffer were considered as mock-plants. The inoculated plants were maintained in an insect-proof glasshouse and observed for symptoms expression till 30 days post inoculation (dpi). Symptom assessment was performed according to Kumari et al. [17], where infected plants started to manifest yellow green mottling patches score were rated as 1, at subsequent stages of infection crinkling appearance (severity grade 2) to spread of patches

and crinkling to newly emerging leaves (grade between 2 to 4 depending on symptom severity).

Detection of PVX transcript by Northern blotting

Total RNA was isolated from 200 mg of tissues obtained from the two uppermost leaves using Trizol (Sigma, St. Louis, USA) as described by Sambrook and Maniatis [31]. The RNA samples (RNA: denaturing mix ratio in 1:3) were electrophoresed in 1.2% denaturing agarose gel and transferred to nylon membrane by capillary transfer method. The RNA was crosslinked with the membrane under UV (1200 × 100 micro joule/cm² energy, 1 min). The CP gene specific probe of PVX was prepared by random priming method and blots were hybridized following standard protocol [31]. Blots were scanned using Phosphor Image analyzer (Fuji, Japan).

Results

Cloning, expression and purification of CaDcr1 from *C. albicans*

RT-PCR of m-RNA isolated from *C. albicans* using the CaDcr1 specific primers resulted in amplification of approximately 1.85 kb DNA fragment. The sequence of the CaDcr1 clone showed 100% identity with the reference sequence. Expression of pET-CaDcr1 in *E. coli* produced an expected size of 69 kDa polypeptide. Initially, most of the CaDcr1 protein fractions were in the pellet fraction as inclusion bodies and only 20% of the CaDcr1 protein appeared in the soluble fraction of the bacterial extract (Fig. 1a). Therefore, the pellet was dissolved in urea buffer and passed through Ni-NTA column to obtain single polypeptide mass of 69 kDa at 120 mM imidazole concentration. In western blot, anti-His antibody detected the 69 kDa protein (Fig. 1b). Approximately, 0.5 mg of soluble recombinant protein was obtained per liter of bacterial culture. Purified fraction of the recombinant protein eluted using 120 mM imidazole concentration in urea solution was refolded through dialysis with decreasing concentration of urea (Fig. 1c) and was further confirmed by MALDI analysis (Supplementary Fig. S1).

Catalytic activity of recombinant CaDcr1

To check the enzymatic activity of the purified CaDcr1, the substrate containing 701-bp segment was prepared by annealing the ³²P labeled plasmid run-off transcript with the nonradioactive complementary transcript (Fig. 2) and in vitro transcripts were hybridized, subsequently incubated with the purified CaDcr1. The assays showed clearly

Fig. 1 Expression and purification of CaDcr1 protein. **a** Pellet (P) and supernatant (S) fractions of the CaDcr1 recombinant protein expressed in *E. coli* BL21DE3 cells induced by varying concentrations of IPTG at 37 °C after 3 h and pET28a (+) was used as induced vector (V) control; **b** CaDcr1 protein collected at imidazole

concentrations (20 and 120 mM) in Ni-NTA affinity chromatography (upper panel) and confirmation of recombinant CaDcr1 protein by western blot (lower panel) analysis using anti-His specific antibody; **c** purified CaDcr1 protein in SDS-PAGE gel (10%) after dialysis. Gel was stained using Coomassie brilliant blue (R-250)

Fig. 2 In vitro transcription of coat protein gene of CMV. Complementary RNAs were produced by transcription of coat protein gene of the CMV isolates cloned in the pGEM-T easy vector in both orientations. Transcript of 701 nt long of anti-sense strand (AUR) and 691 nt long sense strand (PUNE) were generated using Promega Riboprobe Kit. Transcript of pGEM-T easy was used as control. DNA marker of 1 kb was used

that CaDcr1 specifically cleaved the dsRNA of viral origin not the ssRNA (Fig. 3). Cleavage assay using the ^{32} P-labeled transcript in the absence of an annealed non-radioactive sequence did not generate any cleavage site. Hence, this result can be attributed to the presence of short, imperfect and non-specific base-paired intramolecular structures in the transcript. The CaDcr1 recombinant protein could be stored for more than 6 months at -20°C without any detectable loss of activity.

Fig. 3 Degradation of CMV dsRNA by CaDcr1 protein. Viral transcripts (single and double stranded RNAs) were incubated with the recombinant protein and the obtained products were resolved on agarose gel (1.2%). Cleavage of the hybridized dsRNA (In vitro transcript) of CMV by CaDcr1 is shown in the fourth lane from left, whereas lane 1 indicates non-degradation of ssRNA by CaDcr1. Details of other lanes are mentioned on the top of each lane

Allergenicity of the recombinant protein

Before raising transgenic plants for any potential use, it is prerequisite to test allergenicity of the protein and

therefore, the allergenicity prediction of the recombinant CaDcr1 protein was made using SDAP and FARRP softwares. According to these programs, the test protein exhibiting > 35% similarity to a known allergen can be categorized as an allergen. The allergenicity value of CaDcr1 was observed to be within the range of 3–11% which was far below the critical value. Therefore, CaDcr1 protein appears not be a potential allergen (Supplementary Fig. S4).

Development of transgenic tobacco lines over-expressing CaDcr1 and challenge inoculation with PVX

Transgenic tobacco plants were raised to test the efficacy of CaDcr1 for developing resistance against an RNA virus, potato virus X (PVX). A total of 18 lines of *N. tabacum* plants were raised expressing CaDcr1 protein. Seeds of T0 lines were sown on MS media supplemented with 100 mg/L of Kanamycin and the lines that followed proper Mendelian ratio of inheritance (3:1) implying single copy of transgene integration were further advanced to subsequent generations. Consequently, 3 lines of *N. tabacum* were advanced to T2 generation. Total DNA isolated from these plants was subjected to PCR analysis using CaDcr1 specific primers which confirmed presence of the transgene in these plants (Fig. 4).

To check the efficacy of transgenic plants (T2 generation), three lines of *N. tabacum* harboring CaDcr1 of pBIN-CaDcr1 (Lines 3, 5, and 18) were inoculated with sap obtained from PVX infected tobacco plants. It was observed that symptoms started to appear as small patches on the edge of leaves and advanced towards the leaf petioles followed by crinkling of leaves and mosaic like

appearance in the later stages. On *N. tabacum* transgenic plants inoculated with PVX, symptoms started to appear from 12 to 14 days post inoculation (dpi) onwards, whereas on non-transgenic *N. tabacum* plants initial symptoms started from 11 dpi onwards. Symptom severity on both transgenic and non-transgenic plants is presented in the Fig. 5. Transgenic lines NT-3-1, NT-18-1 and NT-18-2 showed delayed symptom expression whereas lines NT-3-2, NT-5-1, NT-5-2 exhibited severe symptoms as compared to other lines. Overall, symptom severity was reduced in transgenic plants as compared to non-transgenic plants.

Relative accumulation of viral RNA and coat protein in transgenic plants

Accumulation of PVX in transgenic lines of *N. tabacum* was tested by Northern blotting. For this, total RNA from the transgenic lines overexpressing CaDcr1 gene and wild type *N. tabacum* plants inoculated with PVX was isolated at 30 dpi. Level of viral RNA in these challenged lines was examined by probing with CP gene of PVX. It was observed that level of accumulation of virus was more in the non-transgenic plants as compared to CaDcr1 expressing transgenic lines. Among the transgenic lines, viral accumulation was found to be the least in line NT-3-1 followed by NT-18-1, NT-18-2, NT-3-2, NT-5-2, and NT-5-1, respectively (Fig. 6).

Discussion

Plant diseases that cause drastic yield loss in crop production are major cause of concern for the over growing world population. Various pathogens such as fungi, bacteria, insects, viruses etc. are primarily responsible for limiting crop production. Plant breeders have developed various effective strategies to provide resistance in crop plants for these pathogens. However, the prevailing methods are time consuming, and in addition, chemical control of plant diseases has various side effects. Among pathogens, viruses are the major cause of concern throughout the world which is responsible for significant yield losses [34] as they generate new variants very fast that makes development of control strategies even more difficult.

The present study developed a novel strategy to generate resistance against plant-infecting RNA viruses through over-expression of a fungal dicer protein from *Candida albicans* (CaDcr1) in tobacco plant. CaDcr1 from recently sequenced *Candida albicans* genome [3] was characterized using bioinformatics tools which indicated conserved characteristics motifs of dsRNA specific ribonucleases. Like other eukaryotic RNase III proteins, CaDcr1 contains a dsRNA binding domain and a nuclease domain [2]. Dicer

Fig. 4 Confirmation of transgenic tobacco lines over-expressing CaDcr1. PCR based conformation was carried out using primers specific to the transgene. *CaDcr1* cloned in plasmid was used as positive control where as non-transgenic *N. tabacum* (NT) and *N. benthamiana* (NB) served as negative controls

Fig. 5 Infectivity of PVX on transgenic tobacco over-expressing CaDcr1. **a** Symptom severity graph following challenge inoculation of transgenic and non-transgenic tobacco lines till 30 dpi. **b–d** Representative photographs of leaves of transgenic lines NT-3,

NT-5 and NT-18 along with mock and non-transgenic (NT) tobacco plants following challenge inoculation with PVX after 30 dpi. Leaves of non-transgenic plant inoculated with buffer are indicated as mock

Fig. 6 Relative accumulation of PVX RNA in transgenic lines. Total RNA was isolated from the upper most leaves of either mock- or PVX-inoculated transgenic or non-transgenic plants (as indicated) at 30 dpi and viral transcript detection was carried out using radiolabeled coat protein gene of PVX as probe. Mock indicates non-transgenic plant inoculated with only buffer devoid of PVX. P1 and P2 indicate two independent representative plants. Transgenic tobacco lines 3-1, 3-2, 18-1, 18-2 indicated less viral load as compared to non-transgenic (NT) and transgenic lines such as 5-1 and 5-2. Ethidium bromide stained gel is used as loading control

belongs to RNase III group of enzymes which is required for generating siRNAs, that control key function in defense against viral infection, transposon silencing and cellular gene repression [7, 24, 38]. The CaDcr1 ORF was cloned

and the expressed to obtain recombinant protein that was purified by Ni-NTA column chromatography. Purified fraction of CaDcr1 protein was further refolded using dialysis by varying the urea concentration. MALDI analysis and western blotting of purified protein conclusively proved the recombinant protein as the CaDcr1 protein. Similarly, PAC I, an RNase III from *S. pombe* also formed inclusion bodies during its expression but methods used by Moore et al. [25] caused 50% of PAC I protein to go in soluble fraction [12, 25], whereas Rnt1p and Bacterial RNase III mostly occur in soluble fraction during purification [19].

RNaseIII proteins such as Rnt1p, PacI, and *E. coli* RNase III required distinct model substrate for its enzyme activity. Rnt1p binds and cleaves its RNA substrates by recognizing NGNN tetraloop and its surrounding element, similarly *E. coli* RNase III binding to substrate and cleavage is enhanced by Mg_2 [21] whereas PacI recognizes long dsRNAs with similar efficiency to *E. coli* RNase III [29] while cleaving short RNA substrates using specific motifs like Rnt1p. In vitro analysis of CaDcr1 proved that it can cleave dsRNA of coat protein gene of CMV, but not the single strand RNA (ssRNA) form which is cleaved by like RNaseA (Fig. 3). Our analysis shows that CaDcr1 can specifically cleave dsRNA substrates, a common activity

among RNase III family enzymes [1]. The results obtained with the recombinant protein indicate that CaDcr1 can function as an enzyme having double stranded-specific RNase activity. However, nature of substrate specificity of CaDCR I and requirement of monovalent and divalent cations for optimal activity needs to be analysed.

Transgenic virus-resistant plants can be obtained by expressing non-viral sequences [28] such as yeast type III endoribonuclease (RNase III), *Pac1* [15], which cleaves only double-stranded RNA (dsRNA), has been shown to confer resistance to plant viruses [35] and to a viroid [32]. Expression in transgenic plants expressing *Escherichia coli* RNaseIII (Rnc) could confer virus resistance [20]. Recently an RNaseIII from *Arabidopsis thaliana* (AtRTL2) has been found to interact with dsRNA molecules derived from viruses and provide resistance by accumulation of small RNAs similar in size to siRNA and hence functions in providing resistance against viruses [16]. Till date, no attempts have been made to express CaDcr1 from *C. albicans* in any plant system. Progeny of these plants over-expressing CaDcr1 showed no morphological changes in transgenic plant that rules out off-target effect of the foreign protein produced in plant. Challenge inoculation of transgenic lines with PVX showed reduced severity of symptoms compared to that of non-transgenic lines while and the level of virus accumulation in transgenic lines compared to that of non-transgenic lines. Among the transgenic lines, viral accumulation was found to be the least in line NT-3-1 followed by NT-18-1, NT-18-2, NT-3-2, NT-5-2, and NT-5-1, respectively which could be either due to site of integration of transgene or level of expression of the CaDcr1. This is the first demonstration of exploiting a fungal dicer CaDcr1 for imparting plant virus resistance.

Taken together, the present study demonstrated the efficacy of the dicer from *Candida albicans*, CaDcr1, as potential candidate to counteract the plant viral infection. In vitro analysis revealed that CaDcr1 cleaved ds RNA of coat protein gene of CMV and when introduced in *N. tabacum* showed some promising results by providing resistance against PVX. The present study provides a new avenue of using fungal dicer for restricting RNA virus infection in plants.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing financial interests.

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