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Short Communication

Genetic machinery for RNA silencing and defense against viruses in Citrus

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Abstract

RNA silencing mechanisms are conserved throughout eukaryotic evolution, possibly due to their importance in viral resistance and other aspects of cell biology. Here, we explored the Citrus EST (CitEST) database in search of sequences related to the most important known genes involved in RNA silencing. Transcripts strongly matching Argonaute (*AGO*), Dicer-like (*DCL*), Hua enhancer (*HEN*), and RNA-dependent RNA Polymerase (*RdRP*) were found in many of the citrus libraries. The reads were clustered and quantified. This shows that post-transcriptional gene silencing apparatus is active in citrus. It seems plausible that a better understanding of the players of RNA silencing in *Citrus* spp. and related genera may help create new tools to defeat the viral diseases that affect the citrus industry. Functional analyses of these citrus genes would enable the pursuit of this hypothesis.

Key words: CitEST, Gene silencing, PTGS.

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RNA silencing is a ubiquitous and highly conserved phenomenon throughout the evolution of eukaryotes (Mette *et al.*, 2000; Baulcombe, 2004). It plays a role in cellular defense against viruses (Waterhouse *et al.*, 2001) and transposons (Bennetzen, 2000), as well as in chromatin remodeling (Chandler and Vaucheret, 2001) and developmental regulation (Kidner and Martienssen, 2005). Three major mechanisms are recognized by which RNA can be silenced in plant cells: i) Post-Transcriptional Gene Silencing (PTGS) mediated by small interfering RNAs (siRNAs); ii) microRNAs (miRNAs) that finely regulate gene expression through mRNA degradation or translation arrest; and iii) Transcriptional Gene Silencing (TGS), which drives chromatin remodeling (via DNA and histone methylation) also through siRNAs (Baulcombe, 2004; Qi and Hannon, 2005). The *trans*-acting siRNA (tasiRNAs) pathway couples two of these mechanisms: a miRNA targeting an intermediate transcript (tasiRNA) that will produce siRNAs to silence target genes (Vazquez *et al.*, 2004). These processes are related and collectively called RNA silencing. They depend upon cellular recognition of double-stranded RNAs (dsRNAs), originated, for example, during virus replication, hairpin RNA or miRNA annealing to a complementary RNA transcript. Detection of dsRNA by cells will elicit the appropriate machinery to degrade any transcripts similar to those found as dsRNA.

The current model of PTGS establishes that once a dsRNA is recognized by the cell, an enzyme called Dicer (coded by four *DCL* genes in *Arabidopsis*) cuts it into 21-23 nt (siRNAs) sections. These fragments are recognized by the so-called RNA-Induced Silencing Complex (RISC), which plays a surveillance role in finding similar transcripts matching one strand of the fragmented RNA, followed by cutting them down or, alternatively, by allowing an RNA-dependent RNA Polymerase (RdRP) to use these siRNAs as primers for extending the cognate transcript, generating new dsRNA that, in turn, will be recognized by Dicer, restoring the cycle in a reiterated process (Benedito *et al.*, 2004). Translation arrest of target transcripts can also occur through this mechanism. Some virus genomes contain genes to counter-attack, called Silencing-Suppressors, which are involved in avoiding silencing mechanisms through strategies, such as hindrance of the RISC assembling or targeting, and avoidance of systemic spreading of silencing signals (Qu and Morris, 2005).

The CitEST database, with 242,790 Expressed Sequence Tags (ESTs), is derived from 33 libraries of eight *Citrus* and one *Poncirus* species (Targon *et al.*, this issue), and represents a valuable platform for analyzing the genetic machinery involved in RNA silencing in citrus species. Identifying RNA silencing will allow a more accurate analysis of these mechanisms, helping to unravel evolutionary aspects. Moreover, mechanisms of gene silencing or silenc-

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ing suppression may be used in citrus molecular breeding, since *Citrus tristeza virus* (CTV; *Closterovirus*), the most important virus of citrus orchards worldwide, holds three distinct silencing suppressors in its ~20-kb genome (Lu *et al.*, 2004). Suppression of endogenous RNA silencing machinery in host cells by multiple mechanisms may represent the basis for virulence and infectivity success. Thus, tackling CTV RNA silencing suppressors may also help to elucidate and identify possible mechanisms of resistance.

In plants, RNA silencing is better understood in model species, such as *Arabidopsis thaliana,* than in crops, and even less in perennial species, such as *Citrus* spp. Some aspects of PTGS have, nevertheless, been investigated in a few woody species, such as *Eucalyptus* (Sassaki *et al.*, 2005), plum (Scorza *et al.*, 2001; Hily *et al.*, 2005) and *Citrus* (Domínguez *et al.*, 2002; Lu *et al.*, 2004; Fagoaga *et al.*, 2006). The CTV coat protein gene *p25* was shown to be silenced under non-selective conditions in a transgenic Mexican lime (*Citrus aurantifolia*) (Domínguez *et al.*, 2002), indicating that the silencing surveillance system is active in this species. More recently, PTGS of the CTV silencing suppressor *p23* was revealed to confer CTV resistance in Mexican lime (Fagoaga *et al.*, 2006), implying how useful this system can be for molecular breeding in order to overcome one of the greatest constraints of the citrus industry worldwide.

This work explored the CitEST database to find genes involved in RNA silencing; transcriptional profiles of *Citrus* and *Poncirus* species were contrasted to establish principles to further functionally investigate this mechanism in citrus. Searches to identify genes that putatively code for proteins known to participate in RNA silencing were carried out using the CitEST database from the Citrus Biotechnology Laboratory at the Instituto Agronômico de Campinas (IAC), Brazil. For this study, 29 libraries encompassing 162,902 reads were used. Searches were performed by tBLASTx using as query protein sequences already characterized and an $E_{value} < 10^{-10}$ as threshold. Reads were scrutinized individually and clustered using the CAP3 software (Huang and Madan, 1999) with default parameters such as an overlap length cutoff of 40 and an overlap length identity cutoff of 80%. Reads composing each tentative consensus (TC) were quantified in selected CitEST libraries and grouped by species and tissues. Since each library presented a different number of reads, the results were normalized to 10,000 reads. Information on construction of the libraries can be found at Targon *et aI*. (this issue).

We found significant matches in the CitEST database for *Argonaute* (*AGO*), *Dicer-like* (*DCL*), *Hua Enhancer* (*HEN*), and the RNA-dependent RNA Polymerase *Suppressor of Gene Silencing* (*SGS3*). No significant matches were found for *SDE2, SDE3, SDE4*, *SGS1 or SGS2*. Quantification of reads assembled into TCs of selected tissues and species can be viewed in Tables 1 and 2, respectively. Table 3 displays sequence similarities among the most abundant citrus TCs and their putative orthologues in *Arabidopsis*.

ARGONAUTE is a family of RNA binding proteins, with a conserved PAZ domain which is implicated in cleaving target RNA through the RNA-induced silencing complex (RISC; Baumberger and Baulcombe, 2005). There are 10 *AGO* paralogues in *Arabidopsis* (Carmell *et al.*, 2002; Morel *et al.*, 2002), and so far, some have been associated with distinct pathways of gene silencing. AGO1 appeared to act with miRNA (Fagard *et al.*, 2000; Vaucheret *et al.*, 2004), whilst AGO4 acted on epigenetic silencing (Zilberman *et al.*, 2003, 2004), and AGO7 and AGO10 on developmental processes (Moussian *et al.*, 1998; Lynn *et al.*, 1999; Hunter *et al.*, 2003). We identified 129 reads significantly similar to *Arabidopsis AGO* paralogues that were grouped into 13 TCs and 18 singlets. Transcriptional modulation of *AGO* TCs within species and tissues was noted. TC1-4 was more actively transcribed than the other clusters; however, detention of differential transcription in tissues or species was limited by the relatively low number of reads in some libraries. It is interesting to note, though, that leaves showed higher expressions of TC1 and TC4, whereas TC2 was the most active in fruit tissues.

Twenty-five transcripts with high similarity to Dicer-like proteins (*DCL1-4*) in the *Arabidopsis* genome (Gasciolli *et al.*, 2005) were grouped into three TCs and seven singlets. A more accurate quantification of gene expression of putative *DCL* paralogues in citrus is required to understand transcriptional modulation in tissues, and environmental and developmental conditions within species, but since the Dicer enzyme is a critical component in RNA silencing mechanisms, this study might raise some special features critical for viral defense.

In *Arabidopsis*, *HEN* was shown to play a role in development (Chen *et al.*, 2002) and virus resistance (Mourrain *et al.*, 2000, 2002). Recently, HEN1 was biochemically characterized and revealed to methylate duplexes of miRNAs or siRNAs to prepare primers for RdRP sequence extension for the following cycle of gene silencing (Yang *et al.*, 2006). Only 14 reads similar to the *Arabidopsis HEN* were retrieved from the CitEST database, corresponding to five TCs and one singlet. Two TCs were formed only by reads from *C. sinensis* (TC1 and 4). Some less represented libraries of specific tissues (such as root, flower and seed) did not show any transcription of *HEN*-related RNA, neither did libraries of some species such as *C. aurantifolia*, *C. limonia*, and *C. latifolia*. Analysis of transcriptional modulation of *HEN*-related sequences seemed to be limited due to the small number of reads found in the CitEST database.

Host RdRPs are thought to act as sensors of foreign RNA, turning them into dsRNAs to enter the silencing pathway (Zamore, 2002). Forty-four reads matching the *Arabidopsis* RNA-dependent RNA polymerase *SGS2/SDE1/RDR6* were retrieved from the CitEST database and they formed six TCs and six singlets. TC1 returned 25

Table 1 - Abundance of reads grouped into tentative consensi (TC) of genes involved in RNA silencing pathways per tissue in selected libraries from the CitEST database[a]. Frequency is given per 10,000 transcripts and absolute numbers of valid reads are shown in parentheses.

	Leaf	Bark	Fruit	Root	Flower	Seed	mean (Σ)
AGO-TC1	1.91 (15)	3.17 (4)	0.81 (5)	-	2.31 (1)	-	**1.53** (25)
AGO-TC2	0.76 (6)	1.59 (2)	2.74 (17)	-	-	2.97 (1)	**1.60** (26)
AGO-TC3	0.64 (5)	-	1.29 (8)	9.64 (2)	-	-	**0.92** (15)
AGO-TC4	1.40 (11)	-	1.45 (9)	-	-	-	**1.23** (20)
AGO-TC5	0.38 (3)	0.79 (1)	-	-	-	-	**0.25** (4)
AGO-TC6	0.13 (1)	-	0.16 (1)	-	-	-	**0.12** (2)
AGO-TC7	0.13 (1)	-	0.48 (3)	-	-	-	**0.25** (4)
AGO-TC8	0.13 (1)	-	0.16 (1)	-	-	-	**0.12** (2)
AGO-TC9	0.13 (1)	2.38 (3)	-	-	-	-	**0.25** (4)
AGO-TC10	-	-	0.32 (2)	-	-	-	**0.12** (2)
AGO-TC11	0.13 (1)	-	0.32 (2)	-	-	-	**0.18** (3)
AGO-TC12	0.13 (1)	-	-	4.82 (1)	-	-	**0.12** (2)
AGO-TC13	0.25 (2)	-	-	-	-	-	**0.12** (2)
Σ	**6.12** (48)	**7.93** (10)	**7.73** (48)	**14.46** (3)	**2.31** (1)	**2.97** (1)	**6.81** (111)
DCL-TC1	0.38 (3)	0.79 (1)	0.48 (3)	-	2.31 (1)	-	**0.49** (8)
DCL-TC2	0.25 (2)	1.59 (2)	0.32 (2)	-	-	-	**0.37** (6)
DCL-TC3	0.25 (2)	0.79 (1)	0.16 (1)	-	-	-	**0.25** (4)
Σ	**0.88** (7)	**3.17** (4)	**0.96** (6)	**0.00**	**2.31** (1)	**0.00**	**1.10** (18)
HEN-TC1	0.13 (1)	-	0.48 (3)	-	-	-	**0.25** (4)
HEN-TC2	0.13 (1)	0.79 (1)	-	-	-	-	**0.12** (2)
HEN-TC3	0.25 (2)	-	-	-	-	-	**0.12** (2)
HEN-TC4	-	-	0.48 (3)	-	-	-	**0.18** (3)
HEN-TC5	0.25 (2)	-	-	-	-	-	**0.12** (2)
Σ	**0.76** (6)	**0.79** (1)	**0.96** (6)	**0.00**	**0.00**	**0.00**	**0.80** (13)
RdRP-TC1	1.40 (11)	1.59 (2)	1.77 (11)	-	-	2.97 (1)	**1.54** (25)
RdRP-TC2	0.25 (2)	-	0.16 (1)	-	-	-	**0.18** (3)
RdRP-TC3	0.13 (1)	0.79 (1)	0.16 (1)	-	-	-	**0.18** (3)
RdRP-TC4	0.13 (1)	-	0.16 (1)	-	-	-	**0.12** (2)
RdRP-TC5	0.13 (1)	-	0.32 (2)	-	-	-	**0.18** (3)
RdRP-TC6	0.25 (2)	-	-	-	-	-	**0.12** (2)
Σ	**2.29** (18)	**2.38** (3)	**2.57** (16)	**0.00**	**0.00**	**2.97** (1)	**2.33** (38)

[a]Libraries considered in this table: Leaf (CA-26-C1-002, CG-32-C1-003, CR-05-C1-100, CR-05-C1-102, CR-05-C1-103, CS-00-C1-100, CS-00-C1-101, CS-00-C1-102, CS-00-C1-401, CS-00-C1-650, CS-13-C1-001, LT-33-C1-003, PT-11-C1-900, PT-11-C1-901; 78,516 reads); bark (CS-00-C2-003, PT-11-C2-300, PT-11-C2-301; 12,610 reads); fruit (CR-05-C3-700, CR-05-C3-701, CR-05-C3-702, CS-00-C3-700, CS-00-C3-701, CS-00-C3-702, CS-00-C3-703, CS-00-C3-704, CS-00-C3-705; 62,003 reads); root (CL-06-C4-500; 2,075 reads); flower (CS-00-C5-003; 4,330 reads); seed (PT-11-C9-005; 3,368 reads). Refer to Targon *et al.* (this issue) for explanation of library codes and total number of sequences in individual libraries.

reads, being by far the most transcribed group, with great activity in *C. aurantifolia*. Such a high expression of RdRP in *C. aurantifolia* is surprising since it is very susceptible to viruses such as CTV in contrast to a relatively low expression in *P. trifoliata* (given the number of reads in this species is not underrepresented), which implies that RdRP in citrus might not be required at high levels to guarantee virus resistance through RNA silencing. Notwithstanding, this enzyme was found to be a key component in antiviral defense mechanisms in plants. The RdRP from *Nicotiana benthamiana* accounted for the high virus susceptibility in this species when its gene was compared with orthologues of close relatives (Yang *et al.*, 2004). Plants of *N. benthamiana* with reduced expression of RdRPs were shown to be more susceptible to viruses with even a greater effect at high temperatures (Qu *et al.*, 2005; Schwach *et al.*, 2005). It is noteworthy, however, that in *C. aurantifolia*, tristeza effects are more pronounced in cool climates, and

Table 2 - Abundance of reads grouped by species into tentative consensus (TC) of genes involved in RNA silencing pathways in the CitEST database[a]. Frequency is given per 10,000 transcripts and absolute numbers of valid reads are shown in parentheses.

	Citrus aurantium	*Citrus aurantifolia*	*Citrus limonia*	*Citrus reticulata*	*Citrus sinensis*	*Citrus latifolia*	*Poncirus trifoliata*	mean (Σ)
AGO-TC1	1.68 (1)	3.02 (2)	-	1.01 (4)	1.27 (10)	-	3.28 (8)	**1.53** (25)
AGO-TC2	-	-	-	2.03 (8)	1.90 (15)	-	1.23 (3)	**1.60** (26)
AGO-TC3	-	1.51 (1)	9.64 (2)	-	1.39 (11)	-	0.41 (1)	**0.92** (15)
AGO-TC4	5.04 (3)	-	-	1.27 (5)	1.39 (11)	1.82 (1)	-	**1.23** (20)
AGO-TC5	-	-	-	-	-	-	1.64 (4)	**0.25** (4)
AGO-TC6	-	-	-	0.51 (2)	-	-	-	**0.12** (2)
AGO-TC7	-	-	-	-	0.38 (3)	-	0.41 (1)	**0.25** (4)
AGO-TC8	1.68 (1)	-	-	-	0.13 (1)	-	-	**0.12** (2)
AGO-TC9	-	-	-	0.25 (1)	-	-	1.23 (3)	**0.25** (4)
AGO-TC10	-	-	-	0.51 (2)	-	-	-	**0.12** (2)
AGO-TC11	-	-	-	0.51 (2)	0.13 (1)	-	-	**0.18** (3)
AGO-TC12	-	-	4.82 (1)	0.25 (1)	-	-	-	**0.12** (2)
AGO-TC13	-	-	-	-	0.13 (1)	1.82 (1)	-	**0.12** (2)
Σ	**8.40** (5)	**4.53** (3)	**14.46** (3)	**6.34** (25)	**6.72** (53)	**3.64** (2)	**8.20** (20)	**6.81** (111)
DCL-TC1	-	-	-	0.25 (1)	0.63 (5)	-	0.82 (2)	**0.49** (8)
DCL-TC2	1.68 (1)	-	-	0.25 (1)	0.25 (2)	-	0.82 (2)	**0.37** (6)
DCL-TC3	-	-	-	0.25 (1)	0.38 (3)	-	-	**0.25** (4)
Σ	**1.68** (1)	**0.00**	**0.00**	**0.75** (3)	**1.26** (10)	**0.00**	**1.64** (4)	**1.10** (18)
HEN-TC1	-	-	-	-	0.51 (4)	-	-	**0.25** (4)
HEN-TC2	-	-	-	-	0.13 (1)	-	0.41 (1)	**0.12** (2)
HEN-TC3	1.68 (1)	-	-	-	0.13 (1)	-	-	**0.12** (2)
HEN-TC4	-	-	-	-	0.38 (3)	-	-	**0.18** (3)
HEN-TC5	-	-	-	0.25 (1)	0.13 (1)	-	-	**0.12** (2)
Σ	**1.68** (1)	**0.00**	**0.00**	**0.25** (1)	**1.28** (10)	**0.00**	**0.41** (1)	**0.80** (13)
RdRP-TC1	-	6.04 (4)	-	1.52 (6)	1.52 (12)	1.82 (1)	0.82 (2)	**1.54** (25)
RdRP-TC2	-	1.51 (1)	-	0.25 (1)	0.13 (1)	-	-	**0.18** (3)
RdRP-TC3	-	-	-	-	0.38 (3)	-	-	**0.18** (3)
RdRP-TC4	-	-	-	0.25 (1)	0.13 (1)	-	-	**0.12** (2)
RdRP-TC5	-	-	-	0.76 (3)	-	-	-	**0.18** (3)
RdRP-TC6	-	-	-	-	0.25 (2)	-	-	**0.12** (2)
Σ	**0.00**	**7.55** (5)	**0.00**	**2.78** (11)	**2.41** (19)	**1.82** (1)	**0.82** (2)	**2.33** (38)

[a]Libraries considered in this table: *C. aurantium* (CA-26-C1-002; 5,950 reads); *C. aurantifolia* (CG-32-C1-003; 6,621 reads); *C. limonia* (CL-06-C4-500; 2,075 reads); *C. reticulata* (CR-05-C1-100, CR-05-C1-102, CR-05-C1-103, CR-05-C3-700, CR-05-C3-701, CR-05-C3-702; 39,481 reads); *C. sinensis* (CS-00-C1-100, CS-00-C1-101, CS-00-C1-102, CS-00-C1-401, CS-00-C1-650, CS-13-C1-001, CS-00-C2-003, CS-00-C3-700, CS-00-C3-701, CS-00-C3-702, CS-00-C3-703, CS-00-C3-704, CS-00-C3-705, CS-00-C5-003; 78,879 reads); *C. latifolia* (LT-33-C1-003; 5,484 reads); *P. trifoliata* (PT-11-C1-900, PT-11-C1-901, PT-11-C2-300, PT-11-C2-301, PT-11-C9-005; 24,412 reads). Refer to Targon *et al.* (this issue) for explanation of library codes and total number of sequences in individual libraries.

disease symptoms may be suppressed at temperatures above 30 °C (Roistacher *et al.*, 1974; Mathews *et al.*, 1997).

A comprehensive transcriptional profiling of genes involved in RNA silencing pathways of citrus through the CitEST project was sometimes impaired by the low number of transcripts strongly resembling characterized genes in other species.

Some efforts have been made in characterizing RNA silencing in citrus, but no information has so far been reported on genetic components in its mechanisms. This work constitutes the first effort in finding the molecular players involved in RNA silencing in citrus species and exploring their relationship with virus resistance. Functional characterization of genes involved in RNA silencing present in resistant and susceptible species to viruses such as

Table 3 - Similarity of citrus tentative consensus with *Arabidopsis* genes involved in PTGS pathways in each species. Only TCs with more than four reads per species were included.

Species	Tentative consensus	Number of reads	Representative read	Putative orthologue	Amino acid identity	Coverage [a]	Score (bits)	E_{value}
C. sinensis	AGO-TC1	10	CS00-C5-003-060-D04-CT.F	AtAGO1	86%	626/1050	1121	0.0
P. trifoliata	AGO-TC1	8	PT11-C2-300-098-F11-CT.F		87%	534/1050	927	0.0
C. sinensis	AGO-TC2	15	CS00-C3-703-002-E02-CT.F	AtAGO4	69%	555/924	791	0.0
C. reticulata	AGO-TC2	8	CR05-C1-100-088-A03-CT.F		70%	698/924	994	0.0
C. sinensis	AGO-TC3	11	CS00-C3-701-102-H04-UV.F	AtAGO5	65%	637/997	819	0.0
C. sinensis	AGO-TC4	11	CS00-C3-700-073-A10-CT.F	AtAGO4	77%	316/924	516	$5e^{-145}$
C. reticulata	AGO-TC4	5	CR05-C3-702-091-E11-CT.F		76%	217/924	353	$2e^{-96}$
P. trifoliata	AGO-TC5	4	PT11-C1-901-030-B06-CT.F	AtAGO9	68%	147/896	225	$3e^{-58}$
C. sinensis	DCL-TC1	5	CS00-C3-701-079-E03-CT.F	AtDCL1	25%	735/1909	305	$6e^{-81}$
C. sinensis	HEN-TC1	4	CS00-C3-705-022-A05-CT.F	AtHEN2	82%	331/995	486	$7e^{-136}$
C. sinensis	RdRP-TC1	12	CS00-C2-003-076-C12-CT.F	AtSGS3	62%	384/625	473	$6e^{-132}$
C. reticulata	RdRP-TC1	6	CR05-C3-700-047-F01-CT.F		54%	604/625	596	$9e^{-169}$

[a]Number of amino acids of conceptually translated TC / number of amino acid of *Arabidopsis* orthologue protein.

CTV may promote the development of molecular tools to overcome one of the most important problems of the citrus industry.

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