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STRUCTURAL-FUNCTIONAL ANALYSIS OF THE PLANT SMALL RNA METHYLTRANSFERASE HEN1

Summary of doctoral dissertation

Physical sciences, biochemistry (04 P)

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This study has been carried out at the Institute of Biotechnology, Vilnius University during 2008-2015.

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VILNIAUS UNIVERSITETAS

SIMONA BARANAUSKĖ

AUGALŲ MAŽŲJŲ NEKODUOJANČIŲ RNR METILTRANSFERAZĖS HEN1 STRUKTŪRINIS-FUNKCINIS TYRIMAS

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CONTENTS

LIST OF ABBREVIATIONS

AdoHcy	S-adenosyl-L-homocysteine; product of AdoMet-mediated methylation reaction
AdoMet	S-adenosyl-L-methionine; cofactor of HEN1
Ago	an Argonaute family protein; Ago subfamily protein
DCL1	*Arabidopsis* Dicer ribonuclease III family homolog Dicer-like 1
dsRNA, dsDNA	double-stranded RNA or DNA
DUF283	domain of unknown function 283
EMSA	electrophoretic mobility shift assay
GST	glutathione S-transferase
HEN1	methyltransferase HUA ENHANCER 1
HYL1	*Arabidopsis* dsRNA-binding protein HYPONASTIC LEAVES 1
L	HEN1 La-motif-containing domain
M	HEN1 methyltransferase domain
miRISC	miRNA-induced silencing complex
miRNA	microRNA; the guide strand of miRNA/miRNA* duplex
miRNA*	the passenger strand of miRNA/miRNA* duplex
P	HEN1 peptidyl-prolyl *cis-trans* isomerase-like domain
PAAG	polyacrylamide gel
PAZ	Piwi-Argonaute-Zwille domain
piRNA	PIWI-interacting RNA
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
R	dsRNA-binding domain
RISC	RNA-induced silencing complex
RLC	RISC-loading complex
RNase III	ribonuclease III domain
SDS	sodium dodecyl sulfate
SE	*Arabidopsis* zinc finger protein SERRATE
SE-core	truncated SE variant consisting of its structured central part
siRNA	small interfering RNA
sRNA	small non-coding RNA
ssRNA, ssDNA	single-stranded RNA or DNA
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

INTRODUCTION

microRNA (miRNA), small interfering RNA (siRNA) and PIWI-interacting RNA are three main classes of eukaryotic small non-coding RNAs (sRNAs), which with members of the Argonaute (Ago) protein family form effector complexes called RNA-induced silencing complexes, or RISCs [1]. miRNA interaction with complementary sequence within mRNA molecule attracts miRISC to target RNA. The Ago protein of miRISC can cleave targeted mRNA or trigger translational repression or/and decay of mRNA. In such a way human miRNAs, which control the expression of more than 60% of protein-coding genes, play an important regulatory role in many processes such as differentiation of tissues and organs, lipid metabolism, secretion of hormones, development and function of immune system, antiviral defence etc [2]. In plants, miRNAs control their growth, morphology, development, sexual reproduction, nutrient homeostasis, adaptation and responses to biotic and abiotic environmental stress, such as salinity, drought stress and pathogen infection [3]. It is therefore not surprising that under stress conditions or during infection, diseases aberrant miRNA expression profiles are detected in organs, tissues, liquids of higher eukaryotic organisms [4]. Bearing this in mind, scientists are trying to develop techniques for fast and accurate detection and diagnosis of various diseases by evaluation of quantitative and qualitative changes in miRNAs. On the other hand, innovative methods are created for treatment of human diseases and for assurance of plants resistance against biotic, abiotic stresses by changing the quantity and function of specific endogenous miRNAs [5, 6]. However, in order to effectively apply miRNA-based technologies in biotechnology and biomedicine, it is necessary to understand thoroughly the molecular mechanisms of miRNA maturation pathway: to identify proteins involved in miRNA biogenesis, to determine their principles of action, the functions "encoded" within the structural domains and to characterize the other processes, which influence the miRNA profile. Although a lot is known about miRNA biogenesis, full mechanismof this process is not completely elucidated.

The biogenesis of plant and animal miRNAs encompasses two main stages. The first is excision of miRNA/miRNA* duplexes from the predecessors (from pri-miRNA and then from pre-miRNA), which is carried out by microprocessor complex composed

of ribonuclease III family enzyme Dicer-like protein 1 (DCL1), zinc finger protein SERRATE and double-stranded RNA-binding protein Hyponastic leaves 1 (HYL1) in plants. At the second stage, after duplex incorporation into Ago, the miRNA* passenger strand is discarded and degraded while the miRNA guide strand remains intact and the miRISC forms. Only the biogenesis of plant miRNAs consists of an additional step between two mentioned above stages - methylation of 3'-terminal nucleotides of miRNA/miRNA* duplexes catalyzed by 2'-O-methyltransferase HEN1, which protects sRNAs from additional 3'-terminal uridylation and/or degradation [7-9]. Although a conservative methyltransferase domain-containing members of HEN1 family are found in animals, fungi and protozoa, however, in contrast to methyltransferases of plants and green algaes, which modify the miRNA/miRNA* and siRNA/siRNA* duplexes, they methylate the single-stranded RNA substrates - piRNA and siRNA molecules [10-13]. Exclusive feature of plants' methyltransferases is a multidomain structure. Primary, secondary and tertiary structures analysis revealed that, besides the conservative methyltransferase domain, the object of the research of this dissertation, *Arabidopsis thaliana* HEN1 contains four additional domains, which are not found in its animal homologues. It consists of a peptidyl-prolyl *cis-trans* isomerase-like domain (P) with unknown function, R^1 and R^2 domains, in which double-stranded RNA binding motifs are found, and located between the latter two a La-motif-containing domain (L) [14, 15]. Despite the fact that this particular methylatransferase is studied in various laboratories around the world, the biological significance of HEN1 and its detailed mechanism of action are still waiting to be elucidated.

Aim of the dissertation - to analyze the mechanism of action of *Arabidopsis thaliana* small RNA methyltransferase HEN1, the functions of its domains and the interactions of the methyltransferase with other proteins involved in plant miRNA biogenesis.

Specific tasks of the dissertation:

1. After evaluation of HEN1 interaction with various RNA and DNA substrates, to determine if methylatransferase specifically binds to miRNA/miRNA* and siRNA/siRNA* classes of the substrates.

2. To identify the domains involved in substrate binding by comparing the affinities of full-length methylatransferase, its mutants and truncated HEN1 proteins to the double-stranded small RNAs.
3. To define a role of HEN1 peptidyl-prolyl *cis-trans* isomerase-like domain P of the unknown function.
4. To test if HEN1 forms complexes with other proteins such as double-stranded RNA-binding protein HYL1, zinc finger protein SERRATE and ribonuclease III family enzyme DCL1, participating in the biogenesis of *Arabidopsis thaliana* miRNA, and to identify protein regions involved in protein-protein interactions.

Scientific novelty. In this work:

- Interaction of HEN1 with single-stranded and double-stranded DNA and RNA substrates was analysed and it was found that the specificity of methyltransferase towards double-stranded siRNA and miRNA molecules is exerted at the substrate binding step. Moreover, it was demonstrated that *in vitro* small non-coding RNA (sRNA) methyltransferase HEN1 exhibits similar binding strength to miRNA/miRNA* and siRNA/siRNA* classes of substrates differing in primary and secondary structure.
- A detailed functional analysis of HEN1 domains was carried out, which demonstrated that methyltransferase and double-stranded RNA-binding domains R^1, R^2 are involved in double-stranded sRNA substrate binding, while a peptidyl-prolyl *cis-trans* isomerase-like domain P, a La-motif-containing domain L and R^2 form contacts with the microprocessor component, a double-stranded RNA-binding protein HYL1.
- It was determined that HEN1 does not interact with microprocessor component zinc finger protein SERRATE, however it forms the contacts with helicase, Piwi-Argonaute-Zwille and double-stranded RNA-binding domains of another component of this complex, ribonuclease DCL1.
- A model of late stage of *A. thaliana* miRNA biogenesis was proposed.

The significance of this dissertation. This work is important both from theoretical and practical point of view. After a detailed functional analysis of HEN1 domains and studies of interaction of methyltransferase with primary (unmethylated) substrates, the

intermediate (hemimethylated) and final (fully methylated) products, a model of HEN1•sRNA/sRNA* complex formation was proposed. The obtained results are important not only for the detailed elucidation of the mechanism of action of *Arabidopsis* HEN1, but also for the determination of general principles of action of methyltransferases of plants, green algae or less analysed animal homologues. In addition, a detailed understanding of the mechanism of HEN1-mediated modification is valuable for the purpose of using methyltransferase for creation of innovative miRNA and siRNA analysis methods, such as methyltransferase-directed transfer of activated groups (mTAG), the technology, which could assist biochemistry, nanodiagnostic and biomedicine specialists to visualize or to isolate or/and enrich sRNA molecules from various biological samples for further experimental or diagnostic purposes. Elucidation of HEN1 interactions with two of three microprocessor components, dsRNA-binding protein HYL1 and ribonuclease DCL1, and the analysis of proteins regions involved in protein-protein interactions, a model of late stage of plants miRNA biogenesis was proposed, which not only fills the gap in miRNA biogenesis, but also ensures its integrity.

METHODS

Plasmids, vectors and system. All DNA manipulations were performed according to the standard procedures [16]. Plasmids, encoding the full-length HEN1 or its mutants containing an N-terminal $(His)_6$ tag within the 20 amino acids polypeptide, were constructed based on pET-15b vector in Department of Biological DNA Modification (Vilnius University, Institute of Biotechnology). Based on pET-43.1a(+) vector, obtained from dr. G. Žvirblis (Vilnius University, Institute of Biotechnology) a plasmid carrying a cDNA of the first double-stranded RNA-binding domain of HEN1 fused with a NusA gene was generated. The pGEX-HYL1, pET28b-SE, pET28b-SE-core and pBA-DCL1 plasmids were gifts from dr. S. Machida and prof. Y. A. Yuan (National University of Singapore). These plasmids and pGEX-5x-1 vector were used for construction of plasmids, which encode the central part of SERRATE (SE) or DCL1, HYL1 mutants fused with glutathione S-transferase (GST) at N terminus. For the protein-protein interaction analysis, a "DupLex-A“ yeast two-hybrid system (OriGene Technologies), provided by dr. A. Gedvilaitė (Vilnius University, Institute of Biotechnology), was used. The pEG202-NLS and pJG4-5 vectors of this system were used to construct the plasmids containing genes encoding HEN1, HYL1, SE and their as well as DCL1 ribonuclease's truncated proteins. For propagation and isolation of plasmids the competent *Escherichia coli* ER2267 cells, prepared as previously described [17], were used.

Expression and purification of recombinant proteins. The synthesis of proteins was carried out at 16 °C for 16-18 h in *E. coli* BL21-CodonPlus(DE3)-RIL cells after addition of 0.1 mM isopropyl β-D-thiogalactopyranoside into LB medium (with exception for GST fused DCL1 domains and SE-core proteins, which expression was induced by addition of 0.4 mM isopropyl β-D-thiogalactopyranoside into M9 minimal medium). His-tagged and GST fused protein were purified using GSTrap HP and HiTrap IMAC HP columns (*GE Healthcare Life science*), respectively.

Preparation of RNA and DNA substrates. RNA and DNA oligonucleotides containing radioactive phosphorus at the 5' end were obtained using T4 Polynucleotide kinase and [γ-^{33}P]-ATP or [γ-^{32}P]-ATP (PerkinElmer). Double-stranded RNA and DNA substrates

were prepared by annealing the labeled oligonucleotide to unlabeled complementary oligonucleotide.

Determination of rate constants of methylation of individual strands in miR173/miR173* duplex by HEN1 under [E]>[S] conditions. Reaction component (A), 0.2 μM double-stranded miR173/miR173* with either guide or passenger strand, ^{33}P-labelled at the 5' end, and 0.5 μM HEN1 in Reaction buffer A [10 mM Tris–HCl (pH 7.4); 50 mM NaCl; 0.1 mg/ml bovine serum albumin] were mixed by a Rapid Chemical Quench-Flow appliance RQF-3 (KinTek) with component (B), containing 200 μM AdoMet in the same buffer. The final concentrations of RNA, protein and AdoMet after combining equal volumes of components A and B were 0.1, 0.25 and 100 μM, respectively. Proteinase K in Stop buffer [7 mM Tris–HCl (pH 7.4); 0.17 mM ethylenediaminetetraacetic acid; 3 mM NaCl;0.5% sodium dodecyl sulfate (SDS)] was added to a final concentration of 0.35 mg/ml to stop the reaction after certain period of incubation at 37°C. Samples were treated with sodium periodate as described previously [18] and resolved on a 13% polyacrylamide gel (PAAG) with 40 % urea. The radioactive bands were then visualized by Fluorescent Image Analyzer FLA-5100 (Fujifilm). The portion of the methylated ^{33}P-labelled strand after each reaction time point were quantified using Multi Gauge v.3.0 software (Fujifilm). Kinetic parameters of miR173 guide and miR173* passenger strands of miR173/miR173* duplex were obtained by fitting experimental data to a single- or two-exponential equation using the GraFit v.5 software.

EMSA. To delineate the binding capacity of proteins towards DNA, RNA substrates, 0.25 μM or 0.5 μM full-length HEN1and 0.25 μM or 7 μM HEN1 mutant proteinwere mixed with 0.05 μM ^{32}P-labelled single- or double-stranded RNA and DNA substrates in the Reaction buffer [50 mM NaCl; 10 mM Tris-HCl (pH 7.4); 5 % glycerol]. For determination of dissociation constants of binary and ternary complexes, a series of 2-fold dilutions of HEN1 or its mutant forms in a range from 250 nM to 0.03 nM or from 3600 nM to 0.44 nM (from 7000 nM to 1.7 nM in thecase of mutant protein HEN1-RHK389,397,477AAA) were mixed with 50 pM ^{32}P-labelled appropriate sRNA/sRNA* duplex in the Reaction buffer with or without 100 μM AdoHcy. In each case, control samples without protein were prepared. After 30 min incubation at 25 °C, the reaction

mixtures were analyzed electrophoretically using the native 8% PAAG. The fractions of bound and free duplex RNA were quantified with Multi Gauge software after the autoradiography. The dissociation constants of binary and ternary complexes were estimated by fitting the data to a singlesitebinding equation using the GraFit software.

Detection of protein–protein interactions via the DupLex-A yeast two-hybrid system. The pEG202-NLS, carrying DNA binding domain (BD, bacterial DNA-binding transcription factor LexA) gene, and pJG4-5, carrying activation domain (AD, 88-residue acidic *E. coli* peptide B42) gene, vectors are used to construct pEG202-NLS-prey and pJG4-5-bait plasmids, which respectively encode the "prey" protein fused with BD and the "bait" protein fused with AD. After passing system control tests, in order to test the possible interaction between "bait" and "prey" proteins, EGY48[pSH18-34] (yeast strain EGY48 holding a LexA operon–LEU2 reporter and transformed with the reporter plasmid pSH18–34 containing the GAL1-lacZ gene under the control of eight LexA operators) cells were co-transformed with pEG202-NLS-prey and pJG4-5-bait plasmids. Also, dissecting the specificity of protein-protein interaction, in parallel, EGY48[pSH18-34] cells were co-transformed with pEG202-NLS-prey and pJG4-5, pEG202-NLS and pJG4-5-bait plasimd pairs. The transformation reactions were spread on minimal agar medium containing the glucose. Then four randomly chosen colonies resulting from each experiment were diluted and spotted on X-gal containing galactose minimal medium with (Gal/+Leu) or without leucine (Gal/-Leu) and grown for 3-4 days. Upon interaction between the "bait" and the "prey", BD and AD are brought in close proximity and a functional transcriptional factor is reconstituted. It activates transcription of reporter *LEU2* and *lacZ* genes, due to which yeast cells can grow on Gal/-Leu and develop a blue color due to hydrolysis of the X-gal. If two proteins do not interact, there is no transcription of the reporter genes and as a result yeast cells grow, but do not turn blue on Gal/+Leu plates and do not grow on Gal/-Leu plates.
resulting from each experiment were

GST pull-down and the immunoblot analyses. One micromolar of GST or GST-fused proteins were incubated with 2 μM of Histagged proteins in the Binding buffer[10 mM Tris–HCl (pH 7.4); 50 mM NaCl; 0.1% Triton X-100; 0.1 mM dithiothreitol] for 20 min at 22 °C. The preformed protein complexes were added to 15 μl Glutathione Sepharose

4B sorbent and incubated for 2 h at 22 °C with constant agitation. Reaction mixtures were washed three times with the Binding buffer. Glutathione Sepharose 4B bound proteins were eluted by heating and were resolved on SDS-PAAG. After being transferred onto Hybond ECL nitrocellulose membrane, it was blocked with 3% bovine serum albumin for 1 h at 22 °C and incubated with primary anti-GST (GE Healthcare Life science) (1:4000) or anti-His (Qiagen) (1:4000) antibodies overnight at 4°C. Antibody-reactive proteins were detected with a horseradish peroxidase-conjugated secondary antibodies after 1 h or 0.5 h of incubation using enhanced chemiluminescence (ECL) western blotting detection reagents (GE Healthcare Life science) or 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate system (Sigma-Aldrich).

RESULTS AND DISCUSSION

The object of the experiments performed in this thesis is a HEN1 methyltransferase of a model plant *Arabidobsis thaliana*. It belongs to a family of small non-coding RNA 3‘–terminal nucleotides 2‘-O-methyltransferases (belong to the sub-subclass EC 2.1.1). Exclusive feature of HEN1 is a multidomain organization. Besides the conservative methyltransferase domain *A. thaliana* HEN1, unlike its homologues in animals [13], has four additional domains (Figure 1A), located in the N-terminal part (Figure 1B): peptidyl-prolyl *cis-trans* isomerase-like domain P, two R^1 and R^2 domains, in which double-stranded RNA binding motifs are found, and La-motif-containing domain L, which interacts with 5'-end of modifiable strand of sRNA/sRNA* duplex [15]. Although the unique structure, the specificity for dsRNA and the biological importance were studied by scientists around the world, the biological significance of HEN1, interaction with substrate and functions of different domains were not elucidated.

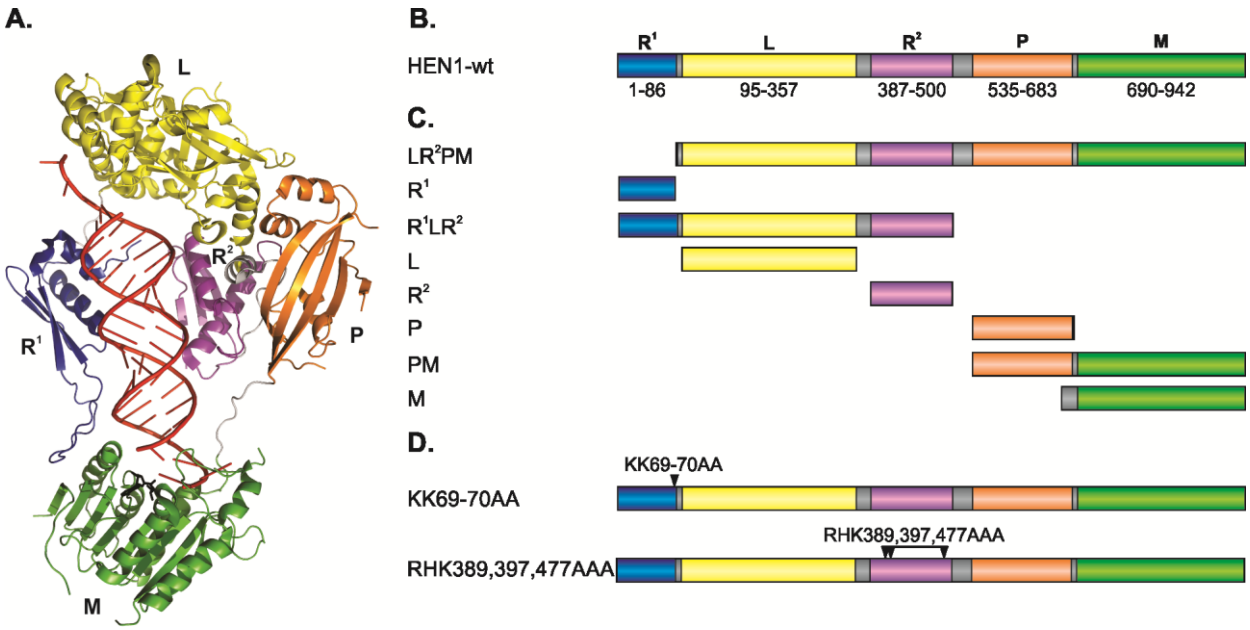

Figure 1. The structure of *Arabidopsis thaliana* HEN1. (A) Representation of domains on the structure of HEN1 in complex with the siRNA type duplex (red) and AdoHcy (black) (PDB ID 3HTX).(B), (C), (D) Domain architecture of full-length methyltransferase (B), truncated HEN1 (C) and proteins with indicated amino acid mutations (D) used in research. R^1 and R^2 (blue and purple, correspondingly): double-stranded RNA-binding domains; L (yellow): La-motif-containing domain; P (orange): peptidyl-prolyl *cis-trans* isomerase-like domain; M (green): methyltransferase domain.

1. Interaction of HEN1 with dsRNA substrates

Not double–stranded DNA or single-stranded RNA, but 21-24 nucleotides long double-stranded sRNA, which are typical in *A. thaliana* [8], are effectively modified by HEN1 *in vitro* [10-12]. We have studied interaction of HEN1 with sRNA/sRNA* duplexes consisting 21-23 nucleotides, with both structurally and in nucleotide sequence identical or different dsDNA and with 21-28 nucleotides long ssRNA or ssDNA (Figure 2A-B) using EMSA method. We found that HEN1 forms complexes only with the dsRNA (Figure 2B-C). Obtained results show that the substrate specificity of HEN1 is determined during binding of sRNA/sRNA duplex, but not during transfer of the methyl group from cofactor AdoMet onto dsRNA.

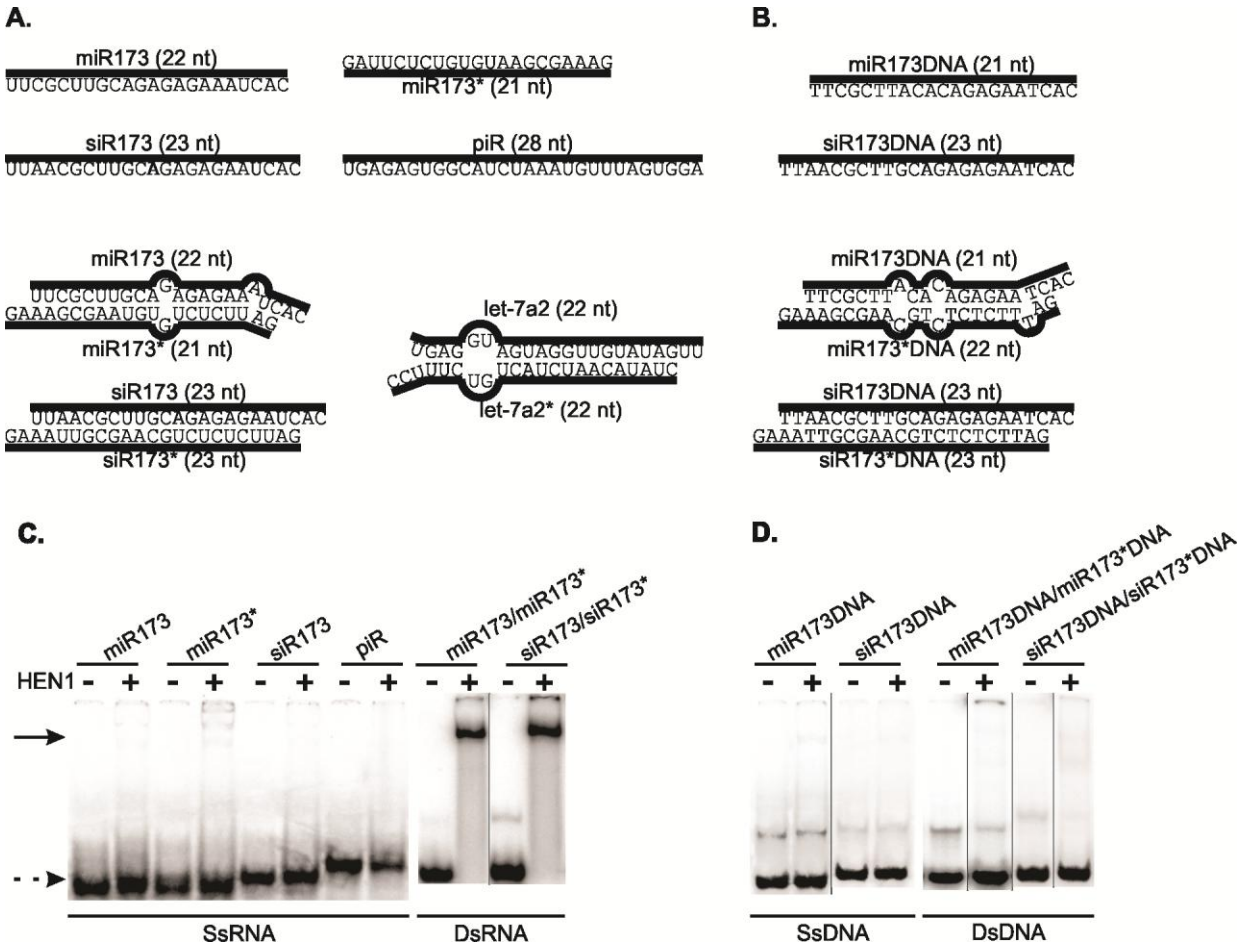

Figure 2. The affinity of HEN1 towards different RNA and DNA substrates. (A), (B) Various RNA (A) and DNA (B) substrates used in the binding assay. (C), (D) Single- or double-stranded RNA (C) and DNA (D) substrates (0.05 μM) were incubated with 0.5 μM HEN1 (+) or in the absence of protein (−) for 30 min at 25ºC and analysed by EMSA. Solid arrow indicates the HEN1 complex with nucleic acids and dotted arrow shows free RNA or DNA.

A. thaliana HEN1 is involved in the biogenesis of miRNA and all types of siRNA [8]. It is noteworthy that miRNA/miRNA* and siRNA/siRNA* duplexes differ not only in primary but also in secondary structure: siRNA type duplexes are composed of perfectly complementary strands, while double-stranded miRNAs have bulges and mismatches. After evaluating the interaction of HEN1 with siRNA type molecule, siR173/siR173*, and with two miRNA/miRNA* duplexes, miR173/miR173* and let-7a2/let-7a2*, differing in nucleotide sequence and structure (Figure 2A, bottom), we found that methyltransferase binds these substrates with similar strength (Figure 3), which lets us assume that HEN1 does not distinguish double-stranded miRNA from siRNA/siRNA* in the cell.

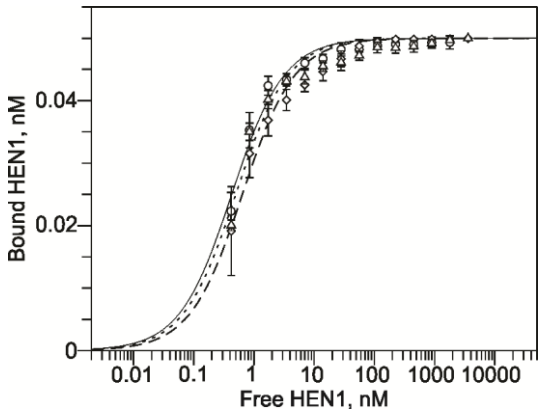

Figure 3. Comparison of HEN1 affinity towards different RNA substrates. A series of 2-fold HEN1 dilutions in range from 3600 nM to 0.44 nM were incubated with 50 pM miR173/miR173* (○, solid line), siR173/siR173* (Δ, short dashes) or let-7a2/let-7a2* (◊, long dashes) RNA for 30 min at 25 °C and analysed by EMSA. Calculated dissociation constants are $K_d^{miR173/miR173*}$ = 0.43 ± 0.05 nM, $K_d^{siR173/siR173*}$ = 0.50 ± 0.05 nM, $K_d^{let-7a2/let-7a2*}$ = 0.64 ± 0.05 nM. Results are means ± S.D.

It is shown *in vitro* that methyltransferase HEN1 modifies both strands of the sRNA/sRNA* duplex [10-12]. It means that one methyltransferase domain-containing HEN1 catalyses the modification of both dsRNA strands only via the intermediate hemimethylated state, when only one strand of sRNA/sRNA* duplex has the methyl group and only then the second strand is methylated (Figure 4). In this case, the unmethylated, hemimethylated and fully methylated dsRNAs should exist in the plant cells.

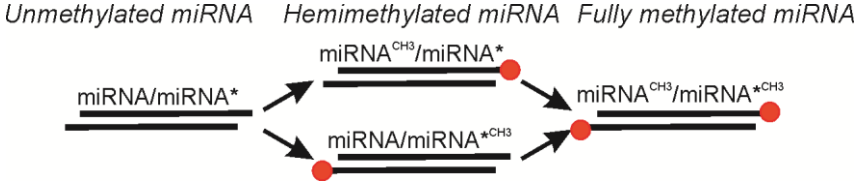

Figure 4. A branched two-step mechanism of miRNA/miRNA* duplex modification by HEN1. Modification of unmethylated miRNA/miRNA* duplex can occur by firstly modifying the passenger strand resulting in the formation of hemimethylated miRNA/miRNA*^{CH3} substrate or by firstly modifying the guide strand producing the hemimethylated miRNA^{CH3}/miRNA* substrate. Red circle indicates methyl group on the 3'-end of RNA strand.

Double-stranded miR173 is a substrate of HEN1 in plants. Using the EMSA method for the evaluation of interaction of HEN1 with the unmethylated miR173/miR173*, hemimethylated miR173^{CH3}/miR173*, miR173/miR173*^{CH3} and fully methylated miR173^{CH3}/miR173*^{CH3} duplexes, we found that binding of HEN1 with fully methylated duplex is approximately 20-fold weaker than with the unmethylated or hemimethylated substrate (Table 1). In the ternary HEN1•sRNA/sRNA*•AdoHcy complex, the 3'-terminal 2'-hydroxyl group of non-modifiable strand of sRNA/sRNA* duplex does not make any contacts with the L domain [15]. Meanwhile, the 3'-terminal 2'- and 3'-hydroxyl groups of complementary strand are fixed by the coordinate bonds in M domain (Figure 5). It means that the only domain, which can be effected by addition of the methyl group to the 3‘-end, is the M domain. We hypothesize that this group causes steric hindrances in the active site of enzyme, which results in change of position of the phosphate of the 3'-terminal nucleotide, therefore neither hydrogen bonds between the phosphate and Arg701 and/or Arg856 of M domain, nor the coordinate bonds between 3‘ terminal nucleotide ribose 2‘- and 3‘-hydroxyl groups and Mg^{2+} (Figure 5) are formed. In conclusion, the obtained results indicate that M domain participates in the substrate binding (Figure 6, right).

Table 1. Interaction of HEN1-wt and its mutant HEN1-RHK389,397,477AAA with double-stranded miR173/miR173*

RNA duplex	Binary HEN1•RNA *K*d (M x 10^{-10})		Ternary HEN1•RNA•AdoHcy *K*d (M x 10^{-10})*
	wt	RHK389,397,477AAA	wt
Unmethylated miR173/miR173*	4.3 ± 0.5	3020 ± 290	3.2 ± 0.3
Hemimethylated miR173/miR173*^{CH3}	4.2 ± 0.6		3.3 ± 0.3
Hemimethylated miR173^{CH3}/miR173*	1.6 ± 0.3		1.7 ± 0.2
Fully methylated miR173^{CH3}/miR173*^{CH3}	72 ± 1	Complex was not identified	18 ± 2

*Reaction mixtures of ternary complexes contained 100 μM AdoHcy. RHK389,397,477AAA denotes mutant HEN1-RHK389,397,477AAA and wt - full-length HEN1. K_d constants were obtained by fitting the data of 3–9 experiments with a single-exponential equation.

AdoHcy is the product of HEN1-catalysed reaction. It is produced upon the catalytic transfer of the methyl group from AdoMet onto 2'-O-ribose of 3'-terminal nucleotide. The binding site of AdoHcy and also of cofactor AdoMet is in M domain of HEN1

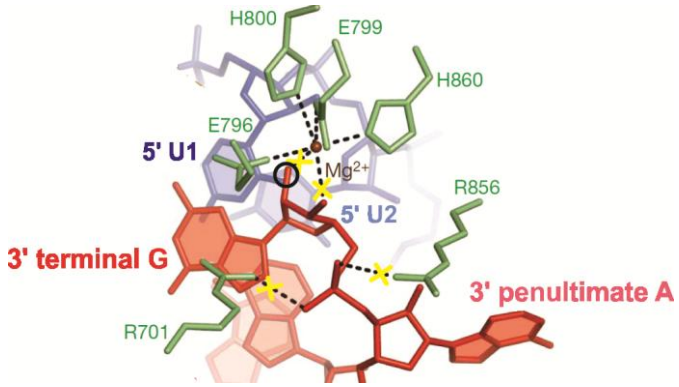

Figure 5. The embedment of the 3'-terminal nucleotide of modifiable strand of sRNA/sRNA* duplex in methyltransferase domain of HEN1 (prepared according to [15]). Both 2- and 3'-hydroxyls of the 3'-terminal nucleotide and four invariant residues, Glu796, Glu799, His800 and His860, of the M domain are coordinated to Mg^{2+}. The backbone phosphate of the 2-nucleotide 3' overhang is hydrogen bonded by Arg701 and Arg856. The modifiable position of 3'-terminal nucleotide ribose is marked by circle. "x" indicates bonds, which can be influenced by methyl group position at 2'-O-ribose of 3'-terminal nucleotide.

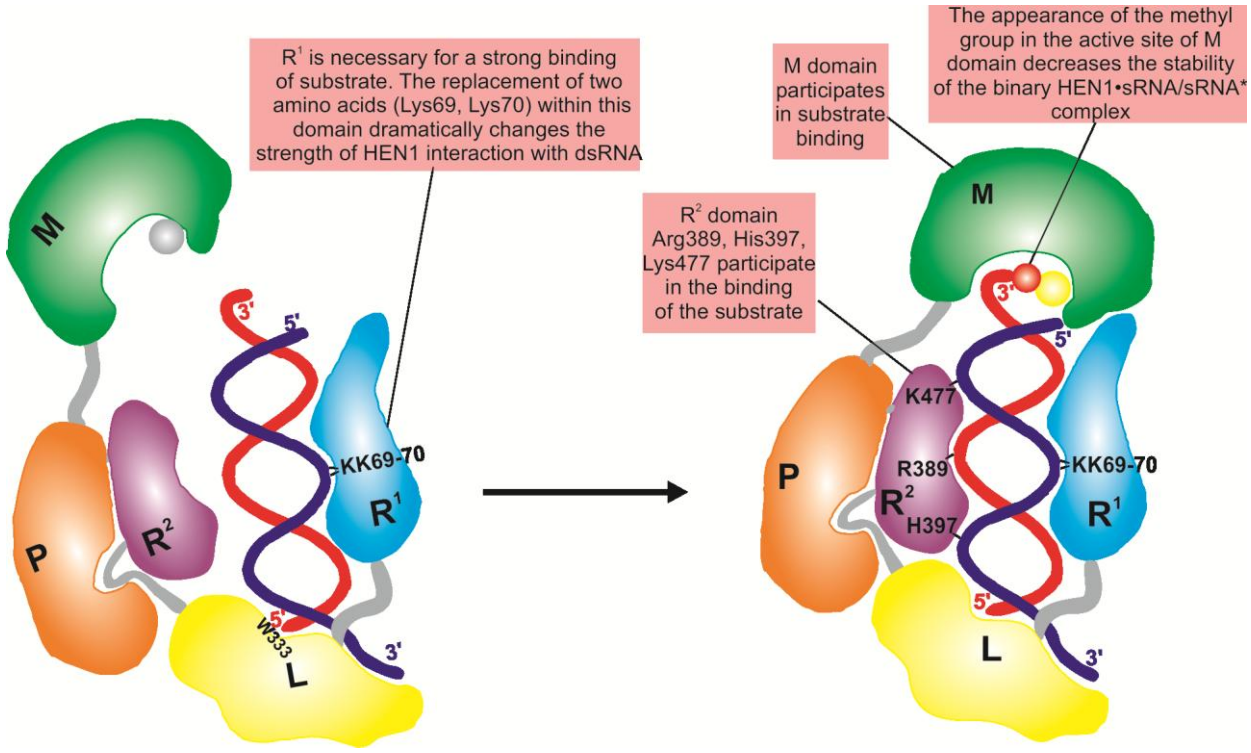

Figure 6. Hypothetical model of HEN1 methyltransferase interaction with sRNA/sRNA* duplex. During the formation of bonds between HEN1 and substrate, initially the first double-stranded RNA-binding domain R^1 (blue) forms contacts with dsRNA, and then the second double-stranded RNA-binding domain R^2 (purple) and methyltransferase domain M (green) contribute to duplex binding. Abbreviations of domains are given in Figure 1. The reaction product AdoHcy is depicted as a yellow circle, the cofactor AdoMet - as a grey circle. Note: methyl group (red circle) on 3'-end of sRNA/sRNA* duplex do not change the sequence of the interaction of domains with the substrate in the supposed model.

(Figure 1A). Thus, having found that methyltransferase's affinity toward miR173/miR173* increases after addition of AdoHcy to reaction mixture (Table 1), we confirmed the importance of M domain in substrate binding (Figure 6, right).

Having studied the interaction of M domain-containing truncated protein HEN1-M (Figure 1C) with miR173/miR173*, we showed that in contrary to full-length methyltransferase HEN1-wt, this protein does not form HEN1•miR173/miR173* complex, which could be detected in the native gel (Figure 7). The obtained results indicate that N-terminal domains of methyltransferase are necessary for the formation of the latter complex.

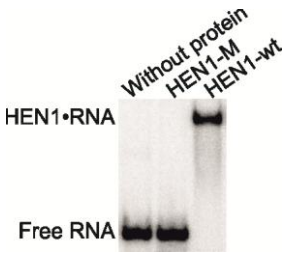

Figure 7. Interaction of full-length HEN1-wt or catalytic domain HEN1-M with the miR173/miR173* duplex. The RNA-binding capacities of both proteins were tested by EMSA using 0.25 μM protein and 0.05 μM dsRNA.

The ternary HEN1•sRNA/sRNA*•AdoHcy complex structure presented in Figure 1A clearly shows that P domain does not form direct interactions with dsRNA. It is known that the L domain is responsible for binding of the 5'-end of modifiable strand of sRNA/sRNA* duplex (Figure 6, left) and together with methyltransferase domain acts as "a molecular ruler" in defining the length of a modifiable substrate [15]. R^1 and R^2 domains hold dsRNA-binding motifs, therefore their participation in substrate binding is assumed. Aiming at better understanding of functions of R^1 and R^2 domains, we examined the mutant proteins of HEN1. We found that HEN1 mutant HEN1-LR^2PM (Figure 1C), lacking the R^1 domain, does not form a stable complex with the substrate in the PAAG (Figure 8, left). Meanwhile the mutant HEN1-R^1 encompassing the R^1 domain (Figure 1C) could bind the substrate, since the free dsRNA band is not detectable in a native gel (Figure 8, left). Aiming at confirmation of importance of the first dsRNA-binding domain in substrate binding, we carried out further EMSA experiments with the HEN1-KK69-70AA mutant, in which lysines of R^1 domain, potentially involved in the interaction with dsRNA, were changed to alanines (Figure 1D and Figure 8, right). The fact that this mutant protein does not form complexes with miR173/miR173* duplex

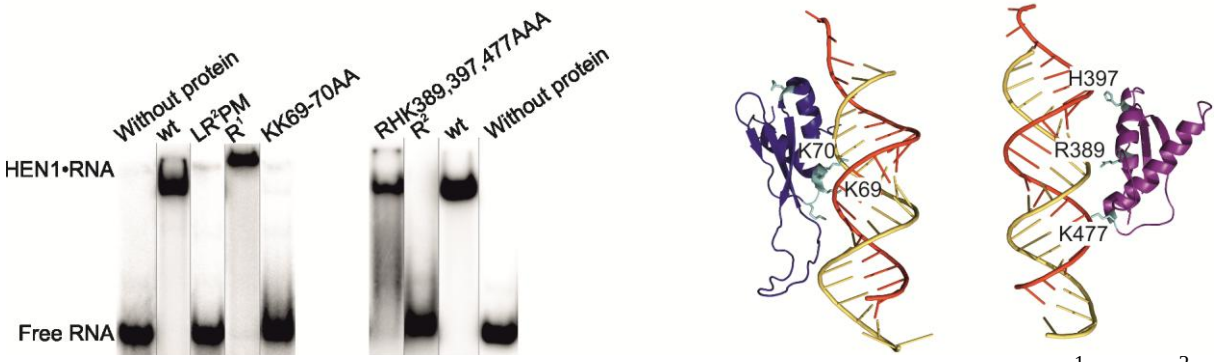

Figure 8. Distinct contributions of the double-stranded RNA-binding domains R^1 and R^2 to miRNA/miRNA* binding. Left, the RNA-binding capacity of the truncated and mutation variants of HEN1 was assessed by EMSA using 0.25 μM protein and 0.05 μM miR173/miR173* duplex. wt denotes full-length HEN1; LR^2PM, R^1, KK69-70AA, R^2, RHK389,397,477AAA – mutants of methyltransferase, which domain architectures are provided in Figure 1C and D. Right, amino acid residues selected for mutagenesis in the dsRNA-binding domains R^1 (blue) and R^2 (purple).

(Figure 8, left) suggests that the first dsRNA-binding domain is essential in formation of HEN1 and substrate complex observable on EMSA-PAAG. Meanwhile, in contrast to domain R^1, the sole R^2 (mutant protein HEN1-R^2, Figure 1C) is not sufficient to form HEN1•miR173/miR173* complexes, detectable in a native PAAG (Figure 8, left). Also replacements of Arg389, His397 and Lys477 within the R^2 domain (Figure 1D) with the alanines, which could potentially change the formation of hydrogen bonds/salts bridges with the bound dsRNA (Figure 8, right), does not dramatically change HEN1 interaction with miR173/miR173* duplex (Figure 8, left). However, although this HEN1-RHK389,397,477AAA mutant forms complexes with substrate, its affinity for dsRNA is approximately 700-fold lower compared to HEN1-wt (Table 1). In conclusion, the obtained results show, that both domains of methyltransferase are important in formation of HEN1 and dsRNA complex, but their input in binding of the substrate is different.

It is notable that HEN1-RHK389,397,477AAA mutant, in contrary to the formation of complex with miR173/miR173* duplex (Figure 8, left), does not form visible complexes with $miR173^{CH3}$/$miR173^{*CH3}$ (Figure 9 and Table 1). The latter duplex differs from the unmethylated one only in the presence of 2'-O-methyl group on both 3'-terminal nucleotides. In the determined ternary HEN1•sRNA/sRNA*•AdoHcy complex structure only 2'- and 3'-hydroxyls of 3'-terminal nucleotide of the modifiable strand of sRNA/sRNA* duplex are fixed by coordinate bonds in M domain (Figure 5) [15]. Thus, methyl group can effect only interactions of M domain with 3'-terminal nucleotide

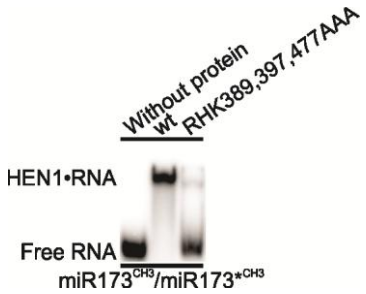

Figure 9. The native gel EMSA of full-length HEN1-wt (0.25 μM) and its mutant HEN1-RHK389,3974,477AAA (7 μM) binding to fully methylated miR173^{CH3}/miR173*^{CH3} duplex (0.05 μM).

(Figure 5). Based on this data, we propose that interaction of the R^2 domain with sRNA/sRNA* helps the methyltransferase domain to find and to position itself at the 3'-end of modifiable strand and the 5'-end of non-modifiable strand of sRNA/sRNA* duplex. Meanwhile, in the process of mutant protein HEN1-RHK389,397,477AAA interaction with fully methylated substrate miR173^{CH3}/miR173*^{CH3} unformed contacts between Arg389, His397, Lys477 and substrate and also steric hindrances, caused by methyl group of 3'-terminal nucleotide appearance, are two factors, which foster M domain movement away from the latter duplex termini and rapid dissociation of the complexes.

Huang and co-authors showed that stacking interaction between L domain Trp333 and the 5'-terminal nucleotide base of the modifiable strand of sRNA/sRNA* duplex (Figure 6, left) is important for substrate recognition, as its substitution with alanine weakens the interaction of HEN1 with sRNA/sRNA* [15]. The role of the L domain in substrate binding is not examined so far detail. It is noteworthy that mutant HEN1-L, encompassing the La-motif-containing domain (Figure 1C), as well as mutants HEN1-M and HEN1-R^2 (Figure 1C, 7 and 8, left), are not sufficient to form HEN1•miR173/miR173* complexes detectable in a native gel (S. Baranauskė and G. Vilkaitis, unpublished results). In this thesis we prove that R^1, R^2 and M domains of HEN1 participate in substrate binding (Figure 6). However, individually only R^1 domain forms complexes with substrate, detectable in the native gel (Figure 7 and Figure 8, left). The affinity of R^1 domain to substrate is only a few times lower compared to that of HEN1-wt (Figure 10 and Table 1), which indicates that the latter domain is necessary for strong substrates binding. It is noteworthy to tell that replacements of only Lys69 and Lys70 within R^1 domain with the alanines are sufficient to prevent the formation of HEN1•RNA complexes, detectable in native gel (Figure 8, left). Based on the obtained data, we propose a model of HEN1 interaction with miRNA/miRNA* and

siRNA/siRNA* duplexes. According to the model, R^1 domain form contacts with dsRNA first (Figure 6, left), and later R^2 and M domains stabilize HEN1•sRNA/sRNA* complex (Figure 6, right).

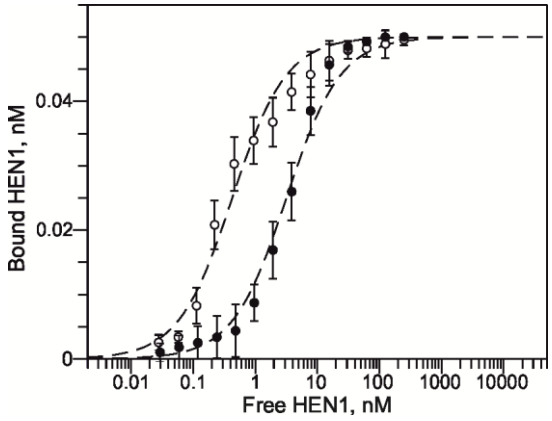

Figure 10. Comparison of full-length HEN1 (○) and its truncated variant HEN1-R^1 (●), representing sole R^1 domain, affinity towards dsRNA. A series of 2-fold protein dilutions in range from 250 nM to 0.03 nM were incubated with 50 pM miR173/miR173* and analysed by EMSA. Calculated dissociation constants of HEN1-R^1•miR173/miR173* complex is 3.2 ± 0.3 nM. Results are means ± S.D.

2. HEN1-mediated methylation of individual strands in miR173/miR173* duplex

In plants methyltransferase HEN1 catalyses the transfer of a methyl group from cofactor S-adenosyl-L-methionine (AdoMet) onto the 2'-hydroxyl of the 3'-terminal nucleotide of miRNA/miRNA* duplex after its excision from miRNA precursor (pre-miRNA). It is known that this modification protects miRNAs from 3'-5' degradation [19]. Then, after the methylation step, double-stranded miRNA together with Ago family protein, usually Argonaute 1 (AGO1), forms pre-miRISC complex, from which the miRNA* passenger strand is excluded and subsequently degraded. As a result, only methylated miRNA guide strands remaining in mature miRISC complex are detected in plants. Although HEN1 efficiently methylates each strand of sRNA/sRNA* duplexes *in vitro* [12], to date it is not clear if the small RNA methyltransferase modifies both strands or only particular strand of mature miRNA/miRNA* duplexes in plants cells.

Suggesting a hypothesis that *in vivo* the degradation of passenger strand of sRNA/sRNA* duplex can be determined by a slower modification of miRNA* strand as compared to that of miRNA guide strand, we calculated the kinetic parameters of HEN1 methylation of each strand of miR173/miR173* duplex,which is naturally methylated by HEN1 in plants, and we compared them with those obtained with hemimethylated

miR173^{CH3}/miR173* and miR173/miR173*^{CH3} substrates (A. Osipenko) (Table 2). The modification rates and amplitudes derived for individual strands of hemimethylated RNA coincide well with the values obtained for miR173/miR173*, which provide compelling evidence that the methylation of the 3'-terminal nucleotide on one strand of a miRNA/miRNA* duplex is not affected by the presence of the 2'-O-methyl group on the 3'-terminal nucleotide of the opposite strand. This data contradicts the hypothetical situation in plants when, for example, (i) after guide strand modification the methyl group in miRNA^{CH3}/miRNA* duplex hinders miRNA* passenger strand‘s methylation; (ii) because of the delay of the passenger strand modification, the hemimethylated miRNA^{CH3}/miRNA* duplex and AGO1 form a complex; (iii) the discarded unmethylated passenger strand is easily degraded, while the remaining methylated guide strand forms the mature miRISC (AGO1•miRNA^{CH3}) complex. Also, contrarily to expectations, according to the model of HEN1-catalysed reaction (Figure 4), the estimated amplitudes values of the rate constants of miR173* passenger strand's modification show that *in vitro* 4/5 of all cases (Table 2) HEN1 modifies the miR173* strand in the HEN1•miR173/miR173* complex during the first round of methylation cycle. The results, the fact that HEN1 binds *in vitro* with miR173/miR173* duplex in orientation that is favorable to methylation of miR173* strand, are confirmed by izotope-partitioning experiments [20]. However, after identification of HEN1 interaction with HYL1 and DCL1 (look for more detailed information in chapter 3), we suppose that in *Arabidopsis* multi-protein complex only miR173 guide strand is modifiable, whereas the 3'-end of the miR173* passenger strand can be shielded by protein(s) (HYL1 or/and DCL1) from methylation and consequently remain intact.

Table 2. Kinetics of HEN1 methylation of unmethylated (miR173/miR173*) and hemimethylated (miR173/miR173*^{CH3} and miR173^{CH3}/miR173*) RNA duplexes.

	miR173/miR173*		**miR173/miR173*^{CH3}**		**miR173^{CH3}/miR173***	
	$k_{obs.}$(min^{-1})	Amplitude (%)	$k_{obs.}$(min^{-1})	Amplitude (%)	$k_{obs.}$ (min^{-1})	Amplitude (%)
Guide strand (miR173)	4.5 ± 0.2	100	7.0 ± 0.2	100		
Passenger strand (miR173*)	35 ± 3 2.9 ± 1	79 ± 3 21 ± 3			38 ± 3 3.3 ± 2	87 ± 5 13 ± 5

All reactions were performed using a fixed 100 μM concentration of AdoMet.The guide and passenger strand methylation data sets were fitted using a single or two-exponential model respectively. The amplitude of the rate constant is expressed as a percentage of the total amplitude

3. Protein-protein interactions of HEN1

The function of HEN1 methyltransferase's P domain homologous to the peptidyl-prolyl *cis-trans* isomerase (PPIase) family was not known until now. Tkaczuk with colleagues ascertained that the peptidyl-prolyl *cis-trans* isomerase-like domain of HEN1 is not responsible for proline *cis-trans* isomerization [14]. Knowing that PPIase-like domains similar to P domain of FK506-binding proteins (FKBP; proteins, which bind the immunosuppressive drug tacrolimus (FK506)) are involved in protein-protein interactions in plants [21], we hypothesize that P domain can be important for the interaction of HEN1 with the other proteins, involved in the miRNA biogenesis pathway.

In *A. thaliana* three miRNA biogenesis proteins, a ribonuclease III family enzyme Dicer-like 1 (DCL1), a double-stranded RNA binding protein Hyponastic Leaves 1 (HYL1) and a zinc-finger protein SERRATE (SE), form a nuclear complex termed microprocessor, which excises 20-22 nt in length miRNA/miRNA* duplexes from their predecessors, primary miRNA (pri-miRNA) and precursor miRNA (pre-miRNA) [22]. The liberated miRNA/miRNA* duplex is recognized and modified by HEN1 methyltransferase. Then methylated duplex forms complex with Ago family protein (usually AGO1), which, after removal of the miRNA* passenger strand, becomes the miRNA guide strand containing argonaute silencing complex called mature miRNA-induced silencing complex (miRISC). It is proven that HYL1 has an influence on which of the two miRNA/miRNA* duplex composing strands will remain in AGO1 owning complex miRISC [23]. Hence, HYL1 participates in miRNA biogenesis not only before the methylation of miRNA/miRNA* duplex, but also after it. This data allowed to suggest a hypothesis that HEN1 through P and/or other domains interacts with the dsRNA-binding protein HYL1.

Using yeast two-hybrid system, we detected the formation of HEN1 and HYL1 complex *in vivo* (Figure 11) and we also showed that HEN1 directly and specifically interacts *in vitro* with HYL1 by GST pull-down and immunoblotting experiments (Figure 12). Until now it was known that HYL1 participates in two stages of miRNA biogenesis: before the excision of the miRNA/miRNA* duplex and after its methylation,

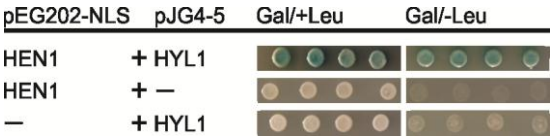

Figure 11. HEN1 interaction with HYL1 in yeast two-hybrid system. On the left, it is depicted, what plasmids with the inserted cDNA of indicated protein or without the insertion („–„) yeast EGY48[pSH18-34] harbours. Gal/+Leu or Gal/-Leu – X-gal containing galactose agarised minimal medium with or withuot leucine respectively.

when HYL1 influences the retention of the miRNA guide strand in miRISC at its formation process [23] and interacts with the protein AGO1 of this complex [24]. It is noteworthy that after confirmation of HEN1•HYL1 interaction with above mentioned methods we proved a continuous double-stranded RNA-binding protein participation in the whole miRNA maturation pathway: beginning from the hydrolysis of pri-miRNA to the end of miRISC formation.

To test the hypothesis that methyltransfrase P domain is involved in protein-protein interaction, using GST pull-down method we studied HYL1 interaction with three truncated variants of HEN1, HEN1-M, HEN1-P, HEN1-PM (Figure 1C), composed of M, P and both domains respectively, using the GST pull-down method. We found that only proteins that possess the P domain interact with the double-stranded RNA binding protein (Figure 12). Aiming to find out if R^1 and/or R^2 and between them located L domain of the N-termini of HEN1 also contribute to the binding of HYL1, we have studied HEN1-R^1LR^2 and HEN1-L proteins (Figure 1C) composed of R^1, L, R^2 domains and of individual L domain, respectively. Both analysed proteins form complexes with dgRNA binding protein (Figure 12). However, in contrast to HEN1-wt or HEN1-R^1LR^2 protein, a much lesser intensity of band of HEN1-L interaction with HYL1, allows to conclude that the L domain is not responsible for the strong interaction of N-terminal part of HEN1 with HYL1. Although using GST extraction method we

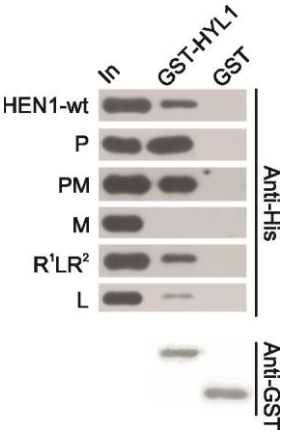

Figure 12. Analysis of protein–protein interaction among HYL1 and HEN1 domains by GST pull-down assay. The experiments were performed using 68 pmol of GST or GST fused HYL1 (GST-HYL1) and 136 pmol of His-tagged HEN1 proteins. The pull-down fractions were analyzed by protein blotting with anti-His antibodies. Presence of GST-HYL1 and GST proteins in pull-down samples was confirmed by immunoblot with anti-GST antibodies. In - input fractions, which represent 20% of the total amount of His-tagged proteins used in pull-down assays. Schemes of truncated HEN1 proteins are depicted in Figure 1.

detected only a non-specific interaction of methyltransferase's R^2 domain with HYL1, however, taking into account that HEN1-R^1 protein, representing the sole R^1 domain, does not form complexes with HYL1 (Figure 13), and the contribution of L domain to the binding is negligible, it could be indirectly assumed that the major determinant of the observed HEN1-R^1LR^2•HYL1 interaction is the R^2 domain. Altogether, our analysis suggests that HYL1 extensively recognizes the central part of HEN1, containing P, R^2 and L domains.

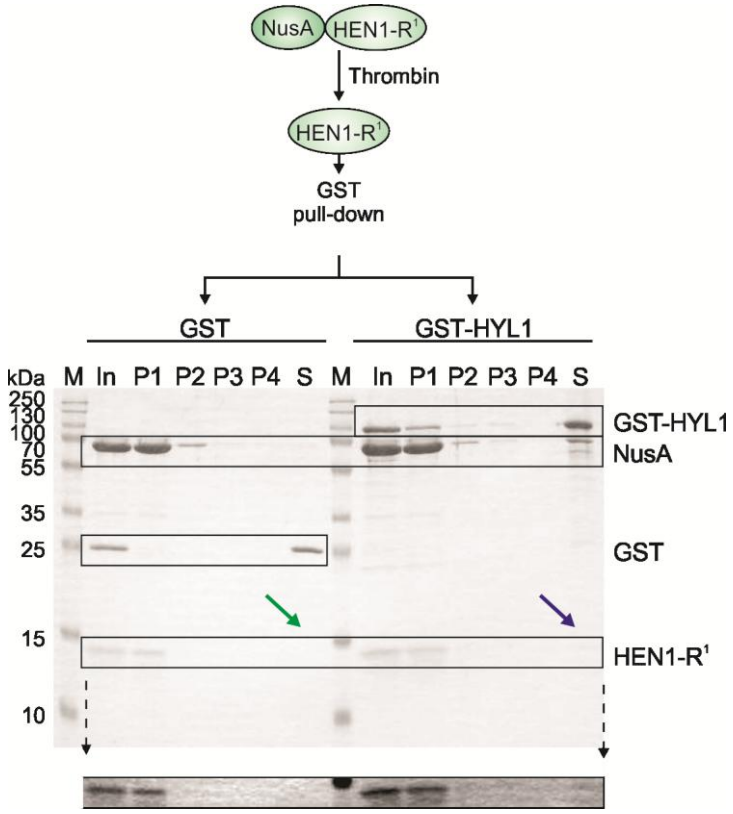

Figure 13. The analysis of interaction between HYL1 and separate HEN1-R^1 domain. The NusA fused R^1 domain was cleaved with thrombin (10 µg baltymo naudota 1 U/µl proteazės) to release R^1 from NusA-tag, which with GST fused HYL1 (GST-HYL1) were used to detect protein's interaction by GST pull-down assay. Boxed areas indicate particular proteins with green and blue arrows pointing to R^1, showing no interaction with either GST or with GST-HYL1, accordingly. This area is additionally highlighted at the bottom of the picture. M – protein molecular weight marker, In – 20% of the total amount of proteins used in pull-down assays, P1-P4 – the amount of proteins that did not bind to GST or GST-HYL1 and were washed away, S – the amount of proteins interacting with GST and GST-HYL1, accordingly.

To identify which of the two R domains of HYL1 interacts with HEN1 protein, we carried out GST pull-down experiments with full-length methyltransferase, its mutant HEN1-P and three truncated HYL1 variants (Figure 14A), containing individual dsRNA-binding domains (R^1 and R^2) each in separate proteins or both combined in a single protein. We have determined that the non-canonical R^2 domain of HYL1 [25] is sufficient for the formation of HEN1•HYL1 complex (Figure 14B). Direct contacts of the P domain of HEN1 with the second double-stranded RNA-binding domain of HYL1 was further confirmed by yeast two-hybrid analysis (Figure 14C). Knowing

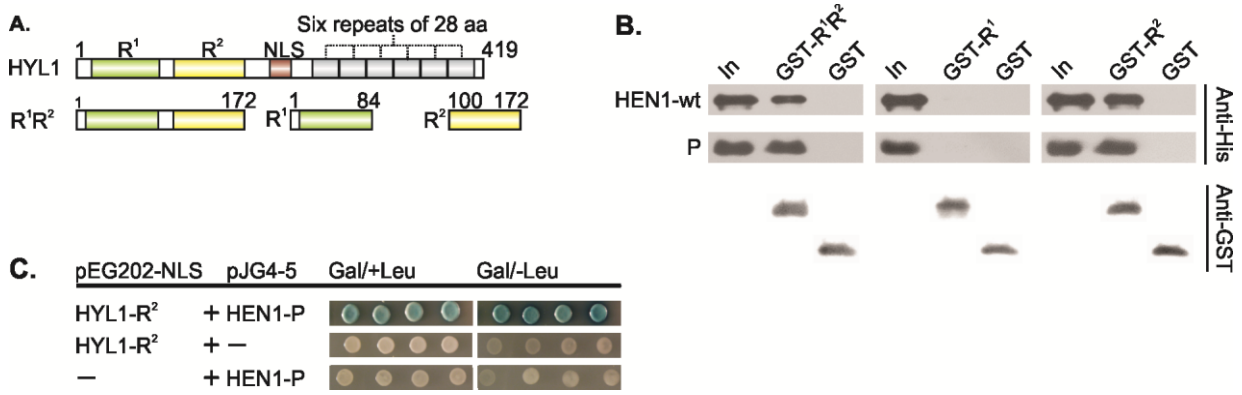

Figure 14. HYL1 interacts with HEN1 via its second dsRNA-binding domain R^2. (**A**) Schematic representation of full-length HYL1 and shorter proteins of separate domains is given (parengta pagal [26]). R^1 and R^2: dsRNA-binding domains, NLS: nuclear localization signal. (**B**) GST pull-down assays and immunoblots indicating HEN1 binds to HYL1-R^1R^2 and R^2 but not to HYL1-R^1. (**C**) Two-hybrid interaction between FK506-binding protein-like domain HEN1-P and the second dsRNA-binding domain HYL1-R^2. All experiments were performed as described in Figure 11 and Figure 12.

that R^2 domain of HYL1 participates in protein-protein interactions with plant microprocessor complex proteins, namely SE and DCL1 [27, 28], it can be assumed that HEN1, SE and DCL1 compete for interaction with dsRNA-binding protein HYL1. Thus, in plant miRNA biogenesis pathway, a certain sequence of formation of interactions between these proteins exists.

To find out if DCL1 and/or SE proteins comprising the microprocessor complex together with HYL1 interact with methyltransferase, we carried out GST pull-down experiments and yeast two-hybrid analysis. We found that HEN1-wt does not interact either with full-length zinc-finger protein SE, or with its truncated variant SE-core, composed of a well-structured part of SE (Figure 15). Meanwhile, an examination of methyltransferase interactions with proteins containing domains of DCL1 showed that helicase, Piwi-Argonaute-Zwille (PAZ) and dsRNA-binding domains form complexes with HEN1 (Figure 16). It is noteworthy that helicase and PAZ domains are also involved in interaction with SE [28], while the domain of unknown function 283 (DUF283) of DCL1, which is essential for interaction with HYL1 [27], is not necessary for formation of HEN1•DCL1 complex.

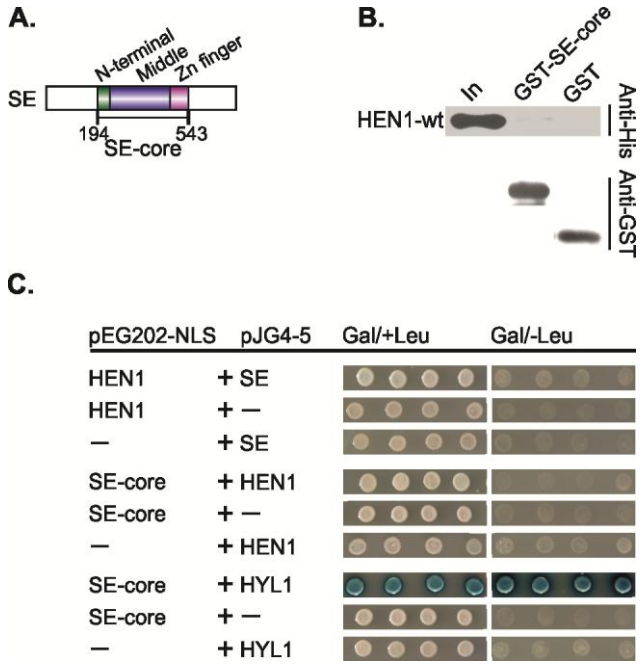

Figure 15. Lack of detectable interactions between SERRATE (SE) and HEN1 methyltransferase. (A) Schematic representation of the domains in SE, which is made according to Machida *et al.* [28]. (B) GST pull-down experiment shows no interaction between central part of SERRATE (SE-core) and full-length HEN1 proteins *in vitro*. (C) Yeast two-hybrid analysis revealed no in-cell interactions between HEN1 and SE. HYL1, a known SE-core interacting partner [28], was used as a positive control for yeast assay. Experiments were carried out as depicted in Figure 12 and Figure 11.

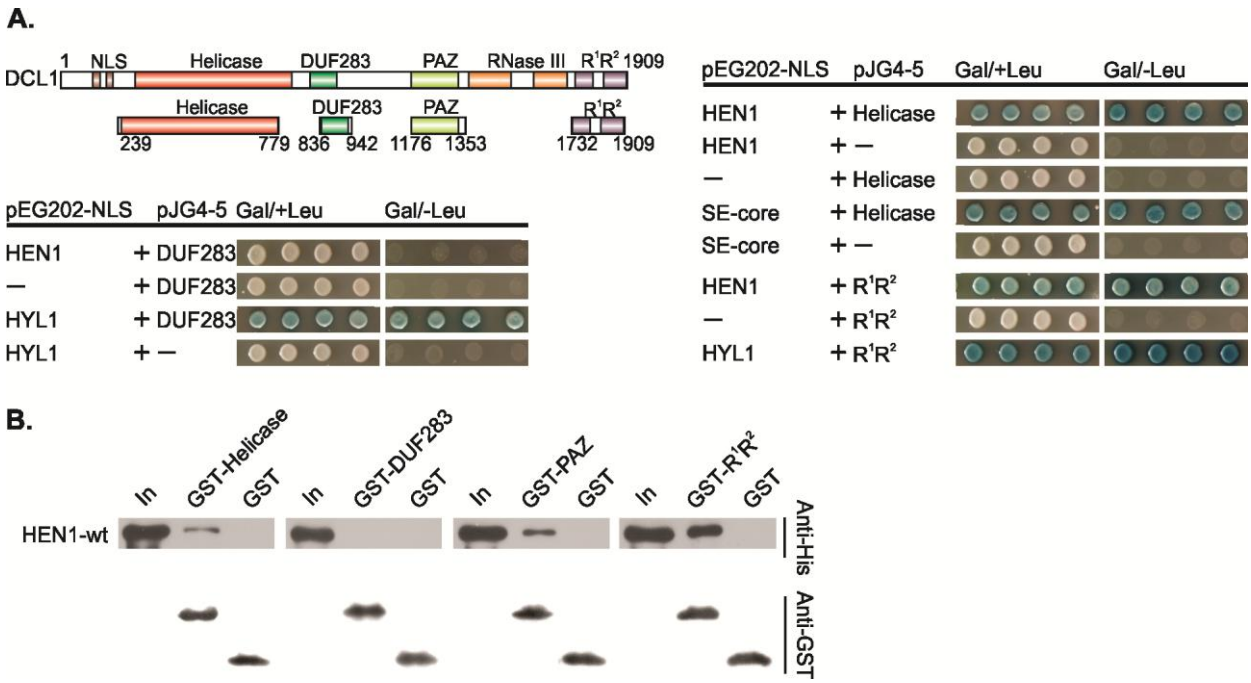

Figure 16. Interaction of HEN1 with individual domains of DICER-LIKE 1 (DCL1). (**A**) Schematic representation of full-length DCL1 and its shorter proteins, which is made according to Mlotshwa et al. [29], used in analysis of protein-protein interaction. Abbreviations of domains are as follows: NLS: nuclear localization signal, DUF283: domain of unknown function 283, PAZ: Piwi/Argonaute/Zwille domain, RNase III: ribonuclease III, R^1, R^2: dsRNA-binding domains. (**B**) Yeast two-hybrid analysis of the interaction between HEN1 and DCL1 domains. Interactions between SE-core with Helicase and HYL1 with DUF283 or R^1R^2 domains of DCL1 served as positive controls [30, 27, 28]. (**C**) Detection of the interaction between DCL1 domains and HEN1 using GST pull-down. Experiments were carried out as described in Figure 11 and Figure 12.

After visualization of network of domain-domain interactions within experimentally determined HEN1•HYL1, HEN1•DCL1 complexes and HYL1•SE, DCL1•SE, HYL1•DCL1 complexes, which were identified by others earlier (Figure 17A), it is clear that both HEN1 and SERRATE physically bind to identical regions of DCL1 (Helicase, PAZ and R^1R^2) and HYL1 (R^2). This finding implies that HEN1 can assemble the particular DCL1•HYL1 complex only when SERRATE is released from the microprocessor complex and disengages its interaction sites on DCL1 and HYL1. Based on collective results, we propose a model in which, following excision of miRNA/miRNA* from primary miRNA by the microprocessor, the HEN1 methyltransferase takes over the place of the SERRATE protein, which leaves the complex (Figure 17B). Then the RNA duplex is methylated and presumably the new HYL1•HEN1•DCL1 complex or this complex without methyltransferase selectively loads the guide strand of the mature miRNA/miRNA* into the RISC complex. This model of late stage of *A. thaliana* miRNA biogenesis was created based on observations obtained by other groups: (i) there is no evidence of SE activity in processes following mature miRNA/miRNA* formation. In contrast, it is shown that HYL1 participates in the precise selection of the guide strand in RISC, the process following miRNA/miRNA* methylation, because, contrary to wild-type *Arabidopsis*, the increase of quantity of passenger strands (miRNAs*) is detected in *hyl1* plants [23, 31]; (ii) the studies in animal systems have shown that the association of the Dicer ribonuclease with the specific dsRNA-binding proteins, TAR RNA-Binding Protein (TRBP) or Protein ACTivator of the interferon-induced protein kinase (PACT) in human or R2D2 (the name of which derives from the fact that it contains two dsRNA-binding domains (R2) and is associated with Dcr-2 (D2)) in flies, is required for directional loading of sRNA/sRNA* duplexes into Ago protein, which ensures preferential retention of the guide strand in RISC [32, 33]. Thus, the heterodimer of ribonuclease and dsRNA-binding protein is necessary not only for effective and precise sRNA/sRNA* duplex excision from predecessors, but it also serves as a platform for mature RISC complex formation. After Fang and Spector have proved that HYL1 interacts with AGO1 protein [24], it is presumable that in plants a complex with similar protein composition does exist, which ensures the miRNA/miRNA* duplex loading into AGO1 in such orientation that the miRNA guide strand will remain in miRISC.

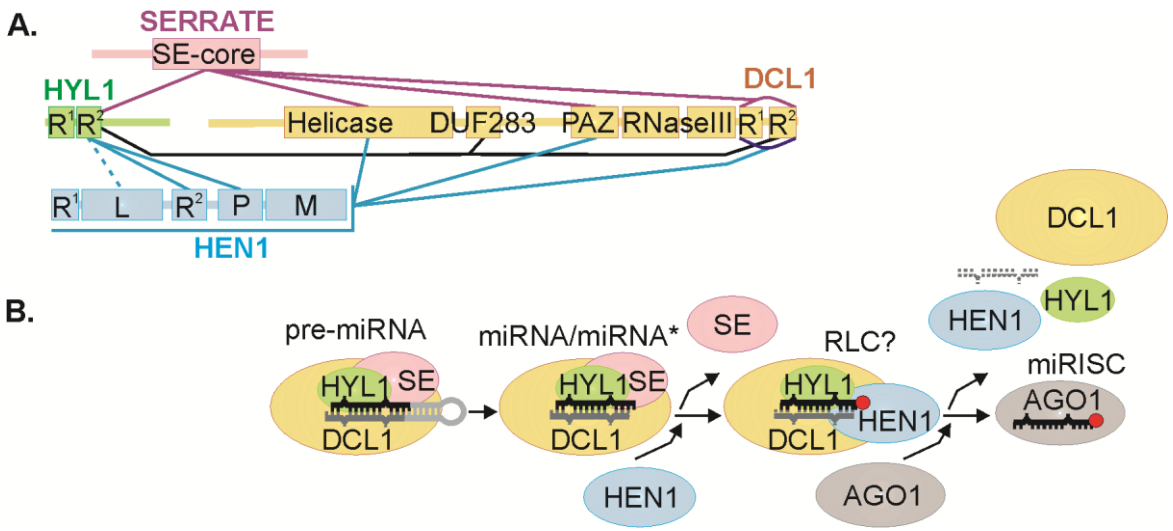

Figure 17. HEN1 interaction network and the proposed model of late stage of miRNA biogenesis. (**A**) Protein–protein interaction network involving SE, HYL1, DCL1 and HEN1. Cyan lines show interactions experimentally determined in this work, purple and black lines depict those reported previously [27, 28, 34]. Abbrevations of proteins' domains are given in Figure 1, 15 and 16. (**B**) Proposed model of miRNA biogenesis envisions that after the miRNA/miRNA* duplex is cut out of its precursor, SE is expelled and HEN1 is bound in the microprocessor complex to form a HYL1•HEN1•DCL1 complex, which might represent the still unidentified plant RISC-loading complex (RLC) anticipated by Eamens et al. [23]. We hypothesize that this complex could direct HEN1 methylation (red circle) to the guide miRNA strand (black) thus labeling it for incorporation into AGO1 complex.

To date it is not clear if the small RNA methyltransferase modifies both strands or only one particular strand of the mature miRNA/miRNA* duplex in a plant cell. Although HEN1 methylates each strand of sRNA/sRNA* duplexes *in vitro* [10-12], we suppose that *in vivo* the HYL1•DCL1 complex sterically orientates HEN1 and thereby predetermines that methyltransferase can only modify the guide strand. Meanwhile the 3'-end of the passenger strand most probably is shielded from methylation and consequently remains intact. Normally, only methylated miRNAs involved in RISC complexes are detected in *Arabidopsis* [10]. It is known that unmethylated strands undergo uridylation by sRNA nucleotidyl transferase HEN1 SUPPRESSOR 1 (HESO1), URIDYLYLTRANSFERASE 1 (URT1) and/or degradation [19]. We speculate that HEN1 marks the correct guide strand of miRNA/miRNA* duplex by methylation whereas HESO1, URT1, which bind to RISC catalytic component AGO1, perform 'quality control' of the small RNA in RISC to ensure that it carries an attached methyl group at ribose 2'-O-position of the 3'-terminal nucleotide. Alternatively, the methylation pattern could determine which strand will be removed during RISC maturation, however, further studies will have to address this intriguing possibility.

CONCLUSIONS

1. Small non-coding RNA (sRNA) methyltransferase HEN1 specifically recognises siRNA/siRNA* and miRNA/miRNA* duplexes at the substrate binding step.
2. Similar dissociation constant values of HEN1 complexes with siRNA/siRNA* and miRNA/miRNA* class substrates revealed that methyltransferase equally well binds sRNA/sRNA* duplexes of different primary and secondary structure.
3. The methyltransferase and double-stranded RNA (dsRNA)-binding domains R^1, R^2 of HEN1 form contacts with double-stranded sRNA.
4. HEN1 R^1 and R^2 domains unequally contribute to the binding of sRNA/sRNA* duplex. A model of HEN1 interaction with sRNA/sRNA* duplex is proposed, according to which at first the double-stranded RNA-binding domain R^1 forms contacts with dsRNA, and later R^2 together with methyltransferase domain stabilize HEN1•sRNA/sRNA* complex.
5. Methyltransferase HEN1 interacts with two microprocessor complex proteins involved in plant miRNA biogenesis: dsRNA-binding protein HYL1 and ribonuclease III family enzyme DCL1.
6. HEN1 peptidyl-prolyl *cis-trans* isomerase-like domain P of previously unknown function interacts with the second dsRNA-binding domain of HYL1. HEN1 R^2 and La-motif-containing L domains contribute to formation of the HEN1•HYL1 complex.
7. Helicase, Piwi-Argonaute-Zwille and double-stranded RNA-binding domains of ribonuclease DCL1 interact with HEN1.
8. HEN1 does not interact with the third microprocessor complex component the zinc finger protein SERRATE.
9. A model of late stage of *Arabidopsis thaliana* miRNA biogenesis is proposed, which envisions that after the miRNA/miRNA* duplex excision from predecessors the microprocessor complex protein SERRATE is substituted by methyltransferase HEN1.

LIST OF PUBLICATIONS

1. **Baranauskė S.***, Mickutė M.*, Plotnikova A., Finke A., Venclovas Č., Klimašauskas S., Vilkaitis G. (2015) Functional mapping of the plant small RNA methyltransferase: HEN1 physically interacts with HYL1 and DICER-LIKE 1 proteins. *Nucleic Acids Res.* 43(5):2802-12.

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2. Plotnikova A.*, **Baranauskė S.***, Osipenko A., Klimašauskas S. and Vilkaitis G. (2013) Mechanistic insights into small RNA recognition and modification by the HEN1 methyltransferase. *Biochem. J.* 453(2): 281-90.

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CONFERENCE PRESENTATIONS

1. **Baranauskė S.**, Mickutė M., Klimašauskas S., Vilkaitis G. Functional mapping of the plant small RNA methyltransferase. "The Non-Coding Genome", Heidelberg, Germany, 2015.10.18-21. Poster presentation.
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3. **Jachimovičiūtė S.**, Plotnikova A., Mickutė M., Osipenko A., Vilkaitis G. Mapping the functional domains of plant HEN1 small RNA methyltransferase. "The 22nd IUBMB & 37th FEBS Congress", Seville, Spain, 2012.09.04-09. Poster presentation.
4. **Jachimovičiūtė S.**, Plotnikova A., Klimašasukas S., Vilkaitis G. Substrate-interacting domains of the small RNA methyltransferase HEN1. "7th Plant Science Student Conference", Halle, Germany, 2011.06.14-17. Oral presentation.
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6. **Jachimovičiūtė S.**, Vilkaitis G, Klimašasukas S. Functional Domains of the small RNA Methyltransferase HEN1. CANGENIN training school "High-troughput Screens in Genome Integrity and Cancer", Oxford, United Kingdom, 2010.05.24-27. Poster presentation.

REZIUMĖ

miRNR, siRNR ir piRNR - mažosios nekoduojančios RNR (mnkRNR), kurios, inicijuodamos RNR-taikinio kirpimą ar transliacijos slopinimą ar/ir suardymą, reguliuoja eukariotinių organizmų biologinius procesus. Augaluose ir gyvūnuose mnkRNR nuo degradacijos apsaugo 3' galinė modifikacija, kurią vykdo HEN1 šeimos metiltransferazės. *Arabidopsis thaliana* HEN1 metilina miRNR/miRNR* ir siRNR/siRNR* dupleksus. Ištyrus šio fermento sąveiką su įvairiais RNR, DNR substratais, nustatyta, kad HEN1 specifiškai atpažįsta siRNR/siRNR* ir miRNR/miRNR* dupleksus substrato surišimo stadijoje. Panaudojus sutrumpintus ir mutantinius HEN1 baltymus su pakeistomis aminorūgštimis, parodyta, kad fermento abu dvigrandinę RNR (dgRNR) surišantys domenai, nors ir nevienodu indėliu, kartu su metiltransferaziniu domenu dalyvauja substrato surišime. Remiantis gautais duomenimis, pasiūlytas HEN1 sąveikos su mnkRNR/mnkRNR* dupleksu modelis. Nustatyta, kad HEN1 į peptidil-prolino *cis-trans* izomerazę panašus domenas, kurio funkcija iki šiol nebuvo žinoma, kartu su La motyvą turinčiu ir antru dgRNR surišančiu domenais sudaro kontaktus su dgRNR surišančiu baltymu HYL1, kuris su ribonukleaze DCL1 ir cinko pirštų motyvą turinčiu SERRATE (SE) ankstyvojoje miRNR biogenezės stadijoje vykdo miRNR/miRNR* dupleksų iš jų pirmtakų iškirpimą. Identifikavus HEN1 sąveiką su HYL1 ir DCL1 bei parodžius, kad HEN1 nesudaro kontaktų su SE, pasiūlytas vėlyvosios miRNR brendimo stadijos procesus paaiškinantis modelis.

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