果 树 学 报 2020,37(7):1080-1088
Journal of Fruit Science

DOI:10.13925/j.cnki.gsxb.20190604

植物病毒来源的小干扰RNA及其在果树病毒研究中的应用

陈 玲,段续伟,张晓明,闫国华,王 晶,周 宇,张开春*

(北京市林业果树科学研究院·农业农村部华北地区园艺作物生物学与种质创制重点实验室·北京市落叶果树工程技术研究中心,北京 100093)

摘 要:RNA沉默是植物中重要的抗病毒机制之一。病毒侵染后,寄主细胞中产生大量病毒来源的小干扰RNA(virus-derived small interfering RNA, vsiRNA),vsiRNA装载到寄主ARGONAUTE(AGO)蛋白中形成RNA诱导的沉默复合体(RNA-induced silencing complex, RISC)靶向病毒基因组,发挥抗病毒作用。近年来,科学家们通过对拟南芥的遗传分析和多种植物vsiRNA的高通量测序,揭示了vsiRNA的起源和生物合成过程。本文综述了vsiRNA的研究现状及其在果树病毒研究中的应用,为果树病毒研究及病毒病防控提供一定的思路和借鉴。

关键词:果树病毒;病毒来源的小RNA(vsiRNA);RNA沉默;vsiRNA应用

中图分类号:S66 **文献标志码**:A **文章编号**:1009-9980(2020)07-1080-09

Small interfering RNA derived from the plant virus and its application in researches on fruit tree viruses

CHEN Ling, DUAN Xuwei, ZHANG Xiaoming, YAN Guohua, WANG Jing, ZHOU Yu, ZHANG Kaichun*

(*Beijing Academy of Forestry and Pomology Sciences/Key Laboratory of Biology and Genetic Improvement of Horticultural Crops (North China), Ministry of Agriculture and Rural Affairs/Beijing Engineering Research Center for Deciduous Fruit Trees, Beijing 100093, China*)

Abstract: Fruit crops belong to perennial woody plants, which may carry viruses during lifetime once they are infected. Fruit tree-infecting viruses accumulate year by year, resulting in reduced tree vigor, fruit yield and quality, and even leading to tree death. Fruit tree virus has a wide host range and multiple transmission pathways, and its spread is accelerated with the frequent transfer of plant stocks. Moreover, fruit tree is usually simultaneously infected with multiple virus species, and the symptom is latent or not easy to be observed. Various differential hosts are usually required to distinguish fruit tree viruses that mostly lack the corresponding herbaceous host. Besides, the amounts of virus in woody plants are small, unevenly distributed, and have seasonal differences. For these reasons, the theoretical and technological researches on fruit tree viruses is relatively backward. However, deep sequencing of small RNA has been successfully applied to the identification of fruit tree virus and the use of hairpin structure to express specific virus double-stranded RNA (dsRNA) for fruit trees to obtain antiviral sources has been studied. In this paper, the mechanism of RNA silencing, the origin of vsiRNA and its biosynthesis process are summarized, which emphatically elaborates the application of vsiRNA in the studies on fruit tree virus, so as to provide ideas and methods for the identification of fruit tree viruses, to acquire fruit tree antiviral resources and to cultivate virus-free stocks. RNA silencing, also known as RNA interfer-

收稿日期:2020-01-10 接受日期:2020-03-29

基金项目:北京市博士后工作经费资助项目(2017ZZ090);北京市农林科学院协同创新专项(KJCX201907-2、KJCX201910);北京市农林科学院博士后基金

作者简介:陈玲,女,博士,主要从事植物病毒研究。Tel:010-62595564,E-mail:chenlingvip1988@126.com

*通信作者 Author for correspondence. Tel:010-82596007,E-mail:kaichunzhang@126.com

ence (RNAi), has been demonstrated as a conserved antiviral mechanism in plants. In plants, RNAi is activated by dsRNA, which is recognized by Dicer-like (DCL) proteins and cleaved into 21-24 nt small RNA. These small RNAs are loaded into RNA-induced silencing complex (RISC) containing ARGONAUTE (AGO) protein for post-transcriptional gene and transcriptional gene silencing. The gene silencing elements of host plants can recognize the dsRNA formed during the process of virus infection and then produce 21-24 nt small RNA, which is the virus-derived small interfering RNA (vsiRNA). Virus infection induces production of a great amount of vsiRNAs, which can be loaded into host AGO proteins to form RISC to target viral genome. In recent years, genetic analysis of *Arabidopsis thaliana* and high-throughput sequencing of vsiRNAs from various plants have revealed the origin and biosynthesis of vsiRNAs. For RNA viruses, vsiRNA is mainly derived from the dsRNA formed by the viral replication intermediate, the dsRNA region formed by the self-complementary pairing of the single stranded RNA of the viral genome, or the dsRNA produced by RNA-dependent RNA polymerase (RDR) that uses viral RNA as a template to replicate. For DNA viruses, research on the source of vsiRNA is less. Until now, vsiRNA production in different plants has not been clearly illuminated, and the known vsiRNA production suggests that vsiRNA production requires the participation of multiple core components of RNA silencing, such as DCL, RDR and AGO proteins. Plant DCL proteins are critical components in the RNA silencing pathway. Different DCL proteins process dsRNA formed during viral infection into specific vsiRNA. The model plant *Arabidopsis thaliana* encodes four DCL proteins, DCL1, DCL2, DCL3 and DCL4, each of which has its own function. DCL1 mainly contributes to the production of miRNA and plays an indirect role in the production of different length of vsiRNA, while DCL4, DCL2 and DCL3 produce siRNA of 21-, 22- and 24-nt, respectively. Although the functions of DCL proteins in the production of vsiRNA are different, they play a complementary and self-balancing role. RDR recognizes RISC cleavage products and converts them as substrates into dsRNA, which once again enter the DCL protein containing cleavage complex to produce secondary siRNA, amplifying the silencing signal. RDR6 has been reported to be a main enzyme involved in the production of secondary small RNA in the cytoplasm. AGO protein is a key component in the RNA silencing effect complex, which binds to vsiRNA to cleave viral RNA, and the cleaved single stranded RNA is the source of secondary vsiRNA. With the development of small RNA sequencing technology, the vsiRNA expression profile of fruit tree viruses has been reported successively. Whereas, the types and functions of endogenous RDR-DCL-AGO homologues involved in vsiRNA production in fruit crops remain unclear, and the studies are rare on the small RNA-mediated antiviral gene silencing pathway in fruit trees. However, vsiRNA has been widely used in the studies on fruit tree viruses based on its origin and distribution characteristics. Specifically, a large amount of vsiRNA can be obtained by deep sequencing of small RNA. Since the sequences of vsiRNA are continuous and overlapped, vsiRNA can be assembled *de novo* into contig through bioinformatics tools for the identification of new viruses, which provides a basis for virus detection. Deep sequencing of small RNA itself can also be used for molecular virus detection, which overcomes the limitations of traditional virus detection methods and can be applied to diagnose fruit tree viruses in a large scale and quickly. This technique plays an important role in virus detection and certification of virus-free plants. In addition, vsiRNA, based on deep sequencing, has also been used to study virus populations in plants. Stem tip tissue culture has been widely used to obtain virus-free plants in production. Current view is that the virus-free stem tip is mainly attributed to its strong RNA silencing ability, suggesting the possibility that the virus-free stem tip or the increased long virus-free stem tip of fruit trees could be obtained through expression of specific vsiRNA. Expression of vsiRNA like the hairpin

RNA has been considered as a feasible technology applied to antiviral genetic engineering for fruit trees to obtain virus-resistant plants. In addition, RNAi induced by artificial miRNA in fruit tree antiviral genetic engineering needs to be further investigated. In conclusion, the detailed description of the application of vsiRNA in the studies on fruit tree virus will contribute to controlling viral disease of fruit trees.

Key words: Fruit tree virus; Virus-derived small interfering RNAs (vsiRNAs); RNA silencing; VsiRNA application

果树是多年生木本植物，一旦感染病毒将终生带毒。由于病毒的逐年累积，树龄越大，发病越重，造成树势衰弱，果实产量和品质下降，严重的造成树死。果树病毒寄主范围广，能侵染多种果树；传播途径多，可通过嫁接、修剪、花粉、昆虫等途径传播，随着种苗的频繁调运，病毒传播更为迅速[1]。此外，果树病毒多为复合侵染，病毒种类多，潜隐或显症时间长，症状不易观察，鉴别寄主种类差异大，往往缺乏相应的草本寄主。此外，病毒在木本植物内的复制积累量低，分布不均且具有季节性差异[2]。

在模式植物和草本植物中，病毒致病性、植物抗病性以及病毒和寄主植物之间的相互作用关系均有研究[3]，与之相比，由于果树病毒研究的复杂性、困难性和长期性，现阶段研究多集中于果树病毒鉴定、病毒检测和病毒脱除，果树与病毒之间的相互作用及果树抗病毒研究相对较少。在模式植物拟南芥以及草本植物中，随着 RNA 沉默机制的深入研究以及在病毒侵染的植物中大量病毒来源的小干扰 RNA（virus-derived small interfering RNA, vsiRNA）的发现，vsiRNA 介导的基因沉默在抗病毒中的作用已被证实。随着生物信息学技术的发展，小 RNA 深度测序已成功运用于果树病毒鉴定，各国果树病毒及其全序列被相继报道。此外，利用发夹结构在果树中表达针对特定病毒的双链 RNA，实现果树抗病毒已有研究[4]。笔者主要综述了 vsiRNA 的研究现状及其在果树病毒研究中的应用，以期为果树病毒鉴定、果树抗病毒资源的获得及无病毒苗木培育提供思路及方法。

1 vsiRNA研究现状

RNA 沉默，也称 RNA 干扰（RNA interference, RNAi），是真核生物中一种保守的基因调控机制。RNAi 具有多种功能，但其最原始的功能是抗病毒防卫功能[5]。植物中，RNAi 由双链 RNA（double-stranded RNA, dsRNA）激活，然后这些 dsRNA 被类 RNase-Ⅲ酶 Dicer-like（DCL）蛋白识别并切割成 21~24 nt 的小 RNA，主要包括小干扰 RNA（small interfering RNA，siRNA）和微 RNA（microRNA，miRNA）。这些小 RNA 被装载到含有 ARGONAUTE（AGO）蛋白的 RNA 诱导的沉默复合体（RNA-induced silencing complex, RISC）中，作用于转录后基因沉默（post-transcriptional gene silencing, PTGS）和转录水平基因沉默（transcriptional gene silencing, TGS）[5]。

依赖于 RNA 的 RNA 聚合酶（RNA dependent RNA polymerase, RDR）可识别 RISC 的切割产物并将其作为底物转化成 dsRNA，从而再一次进入 DCL 蛋白切割复合体，产生次级 siRNA，实现沉默反应信号的放大，沉默信号的放大也与 RNA 沉默信号的传导有关[6]。细胞自主性 RNA 沉默过程中形成的 21~24 nt 小 RNA 可通过胞间连丝传到相邻细胞。此外，沉默信号也能通过韧皮部进行长距离的系统传播，移动的小 RNA 分子同样能激活 PTGS 和 TGS[6]。

细胞质中的 RNAi 可由异质 RNA 或内源核酸水解切割的 RISC 片段激发。对寄主植物而言，植物病毒基因组是一类异质 RNA，寄主的基因沉默机制能识别病毒侵染过程中形成的 dsRNA 进而产生 21~24 nt 的小 RNA，这类外源小 RNA 则是 vsiRNA。vsiRNA 也是最早被发现的一类小 RNA，现已证实 vsiRNA 能装载到寄主 AGO 蛋白中以碱基互补配对的方式靶向病毒基因组。

病毒 dsRNA 的形成是寄主抗病毒基因沉默的诱发子。病毒形成 dsRNA 的途径有多种。对于 RNA 病毒，vsiRNA 主要来源于病毒复制中间体形成的 dsRNA、病毒基因组单链 RNA 自身互补配对形成的 dsRNA 区域或者 RDR 以病毒 RNA 为模板形成的 dsRNA[7]。对于 DNA 病毒，vsiRNA 来源的

研究较少。双链DNA病毒花椰菜花叶病毒（cauliflower mosaic virus, CaMV）复制过程中产生35S RNA的复制中间体，其5’末端翻译前导区的大量次级结构是vsiRNA产生的主要来源[8]。具有单链DNA基因组的双生病毒在复制过程中并未形成dsRNA，在侵染寄主植物过程中产生的vsiRNA可能来源于转录过程中重叠区域的互补配对或RNA转录物中的内部折叠区[9]。

不同植物中vsiRNA的产生过程尚未详细阐明，目前已知的vsiRNA产生过程都是建立在对拟南芥的遗传分析和对多种植物vsiRNA表达谱的分析基础之上，这些结果表明vsiRNA的产生与植物内源小RNA的产生十分相似，都需要DCL、RDR以及AGO蛋白等多种RNA沉默核心组分的参与。

植物DCL蛋白是RNA沉默途径中至关重要的组分，不同的DCL蛋白将病毒侵染过程中形成的dsRNA加工成特定的vsiRNA。模式植物拟南芥编码4个DCL蛋白，分别是DCL1、DCL2、DCL3和DCL4，每个DCL蛋白都有其各自的功能。DCL1主要有助于miRNA的产生，在不同vsiRNA的产生中发挥间接作用，而DCL4、DCL2和DCL3分别形成21-、22-和24-nt的siRNA[10]。DCL蛋白在vsiRNA产生过程中的功能不同，但彼此通过功能互补和自我平衡的方式发挥作用。正链RNA病毒主要激发21-和22-nt的vsiRNA[11-12]。但DCL4倾向于细胞内抗病毒反应，而DCL2作用于细胞间的抗病毒反应[13]。有关植物DNA病毒产生vsiRNA的研究还很局限，植物DNA病毒vsiRNA的生成途径与RNA病毒差异很大，现阶段的研究认为DNA病毒vsiRNA的产生需要四种DCL蛋白的参与，可以激发不同种类的vsiRNA产生[14-15]。

病毒侵染的植物体内存在两类vsiRNA，一类是病毒侵染过程中形成的dsRNA被植物DCL蛋白直接切割形成的初级vsiRNA，另一类是次级vsiRNA。次级vsiRNA的产生依赖RDR沉默信号的放大。这种沉默信号的放大过程需要siRNA引导AGO1切割病毒的转录物，切割产物在SGS3/SDE5/RDR6的作用下加工成dsRNA，然后dsRNA作为DCL的底物被切割产生次级vsiRNA[16-17]。寄主RDR蛋白是参与vsiRNA产生和抗病毒的重要组分[18]。其中，RDR6是细胞质中与次级小RNA产生有关的主要酶类[19]。

AGO蛋白是RNA沉默效应复合物中的关键组分，是RNase-H类型的核酸内切酶[16]，AGO蛋白结合vsiRNA切割病毒RNA，然后被切割的单链RNA就是依赖RDR产生的次级vsiRNA的源头。AGO家族成员的数量在不同植物间存在差异，这些AGO蛋白在抗病毒防卫反应中的功能不同，有时也有功能冗余。

近几年，随着小RNA测序技术的发展，果树病毒vsiRNA表达谱的研究已有报道。柑橘黄脉病毒（citrus yellow vein clearing virus, CYVCV）侵染柠檬后的visRNA主要集中在21-和22-nt，推测柑橘上存在类似于拟南芥中DCL4及DCL2的同源物[2]。双生病毒柑橘褪绿矮缩病毒（citrus chlorotic dwarf virus, CCDV）侵染柠檬后，vsiRNA长度以22-nt为主，其次为21-nt，再次为24-nt，表明可能是柑橘内源DCL2、DCL4及DCL3同源物共同参与了柑橘褪绿矮缩病毒vsiRNA的合成[2]。来源于苹果茎沟病毒（apple stem grooving virus, ASGV）的vsiRNA也主要以21-和22-nt为主[20-21]。目前，果树上与vsiRNA产生有关的内源RDR-DCL-AGO同源物的种类及其功能还不清楚，小RNA介导的抗病毒基因沉默通路相关的研究很少，后续需要深入研究。

2　vsiRNA在果树病毒研究中的应用

2.1　新病毒的鉴定

小RNA深度测序可获得大量vsiRNA，因vsiRNA在序列上是连续且重叠的，通过生物信息学工具可将vsiRNA进行从头组装成contig，用于发现和鉴定新病毒或者新株系[22-24]。该方法已应用于果树病毒资源鉴定[25-26]，并成功鉴定和组装出多种果树病毒。Ruiz-Ruiz等[27]利用vsiRNA重新组装出T318A西班牙柑橘速衰病毒（citrus tristeza virus, CTV）分离物的全基因组序列。在患有重要经济病害——红斑点病的葡萄上，科学家组装得到了一种新的DNA病毒和两个类病毒，从而进一步证明以vsiRNA为基础的深度测序方法可以从头组装出DNA或RNA病毒基因组及其变异种[28]。对健康的和被侵染的柠檬香果植物进行小RNA深度测序，一种新病毒柑橘脉突病毒（citrus vein enation virus，CVEV）被鉴定为柑橘脉突病害的致病病原[29]。利用vsiRNA深度测序在葡萄和苹果中鉴定得到了新的类病毒[30]。近5 a（年）来，许多新的果树病毒被陆

续鉴定，如猕猴桃褪绿环斑相关病毒（Actinidia chlorotic ringspot-associated virus, ACRSaV）、山核桃花叶相关病毒（pecan mosaic-associated virus, PMaV）、苹果坏死花叶病毒（apple necrotic mosaic virus, ANMV）、苹果双生病毒（apple geminivirus, AGV）、李属 T 病毒（prunus virus T, PrVT）、杏脉明相关病毒（apricot vein clearing-associated virus, AVCaV）、桃叶痘伴随病毒（peach leaf pitting-associated virus, PLPaV）等[31-37]。

2.2 病毒分子检测

RT-PCR、多重 PCR 以及实时荧光定量 PCR（real-time fluorescent quantitative PCR, RT-qPCR）等技术是检测果树病毒的常用方法[38-40]。而通过小 RNA 测序获得的新型病毒或病毒的不同分离物和株系，能全面揭示寄主植物体内病毒的变异情况[41]，有利于病毒生态学[42]和病毒种群[43]的研究，更重要的是为病毒检测提供了依据，有利于更精准地设计针对某种病毒的特异性检测引物。例如，利用 vsiRNA 测序，从嫁接到酸橙上的甜橙中得到了 SG29（强毒）和 Bau282（中性）两个西西里岛上 CTV 种群中的代表性分离物。地中海分离物和外来分离物之间系统发育关系表明 SG29 聚合到“VT-Asian”亚型中，而 Bau282 则属于 T30 分支中[44]。基于深度测序的 vsiRNA 组装的准确性对分析软件的依赖性较高，科学家已开发出多个具有不同应用程序的计算机软件，如 ViVan[45]、ViVaMBC[46] 和 LayerCake[47] 来分析病毒 vsiRNA 深度测序的数据。此外，小 RNA 深度测序本身也可用于病毒检测，该方法克服了传统病毒检测方法的局限性，能大规模且快速诊断植物病毒[48]。利用这种技术能同时检测植物样品中已知的、未知的、含量高的及含量低的几乎所有 RNA 病毒、DNA 病毒以及类病毒。对于以无性繁殖材料为主的果树，小 RNA 测序能检测母株、收集的原种、苗圃以及保存的种质资源中是否携带病毒[48-50]。因此，小 RNA 测序能作为病毒检测的一种手段，完善和简化现有病毒检测体系，在病毒检测和无病毒苗木认证过程中发挥重要作用。研究表明，小 RNA 测序能成功检测经药剂病毒唑处理自然携带葡萄卷叶病毒 1 号（grapevine leafroll-associated virus 1, GLRaV-1）、葡萄斑点病毒（grapevine fleck virus, GFkV）和沙地葡萄茎痘伴随病毒（grapevine rupestris stem pitting-associated virus, GRSPaV）的葡萄植株的带毒情况[50]。Vigne 等[51]比较了双抗体夹心酶联免疫吸附测定（double-antibody sandwich enzyme-linked immunosorbent assay, DAS-ELISA）、RT-qPCR、免疫捕获 RT-PCR、小 RNA 测序、转录组测序以及 vsiRNA 印迹共 6 种检测方法对葡萄扇叶病毒（grapevine fanleaf virus, GFLV）的检测情况，明确了小 RNA 测序在无病毒苗木认证方面的应用前景。

2.3 病毒脱除与抗病毒基因工程

目前，欧美各国在明确病毒种类的基础上，制定了较完善的病毒病防治措施，并已经证明栽培无病毒苗木既能有效地防治果树病毒病的危害，也能显著提高果品产量和品质，是果树病毒病防控过程中一项行之有效的技术。获得无病毒苗木的方法有多种[52]，而茎尖组织培养获得无病毒苗木的技术已在生产实践中广泛应用。现阶段的研究认为茎尖脱毒的主要原因是其具有很强的 RNA 沉默能力，在恢复无毒的茎尖组织中，vsiRNA 的含量相对较高[53-54]。果树潜隐病毒 ASGV 侵染梨茎尖后的 vsiRNA 分布主要集中在 21-和 22-nt，对 24 ℃和 37 ℃分别处理的梨茎尖 vsiRNA 进行分析发现，37 ℃条件下，vsiRNA 的积累量增加，RNA 沉默核心组分的表达量上调，病毒含量下降，表明热处理脱毒的原理可能是茎尖 RNA 沉默作用增强[21]，茎尖脱毒组织中 vsiRNA 积累增多则暗示了可通过表达特异的 vsiRNA 实现果树茎尖无毒，亦或使得无病毒茎尖的长度增加，有助于获得脱毒植株。

RNAi 在植物抵抗病原物侵染中发挥着重要作用。除了参与 vsiRNA 产生的细胞组分（如 DCL、RDR 和 AGO）外，越来越多的实验证据表明植物中 vsiRNA 自身也在抗病毒免疫反应中发挥着直接作用。但自发的 vsiRNA 诱导的抗病毒反应并不足以抵制病毒侵染。因此，可通过表达 vsiRNA 的方法人工诱导 RNAi，其中发夹 RNA（hairpin RNA, hpRNA）和人工 miRNA（artificial miRNA, amiRNA）技术已被广泛应用于植物抗病毒基因工程[55-56]。在病毒基因组序列及内含子形成的发夹结构中，dsRNA 可以很好地表达，其激活的 PTGS 具有很高的沉默效率[57]。传统育种方法在果树抗病毒育种中面临很大的挑战和限制性因素，实现果树抗病毒，转基因是较为可行的办法[58]。较早的策略是将病毒基因组的一段序列插入植物基因组中，实现对病毒本身的

抗性，该方法也称为病原物来源的抗性（pathogen-derived resistance, PDR）[59]。利用该方法获得的一些抗病植物已成功商业化，也有的还处于申报许可过程中，主要转化的是编码病毒衣壳蛋白的基因，果树中主要包括李子、番木瓜、柑橘和葡萄[58]。然而，对PDR的机制研究表明，多数情况下观察到的植物抗病毒表型并非是由病毒蛋白介导的，而是诱导了RNAi抗病毒机制[58, 60]。RNAi信号能从砧木传递到接穗[61]，这就为通过RNAi技术培育抗病毒砧木，并且嫁接亲和接穗品种提供了可能，该技术的优势是培育的转基因砧木能使不同的接穗品种抗病毒。此外，接穗品种中没有转基因成分，使得该技术从生物安全方面考虑也具有可行性[62]。目前已有研究表明通过RNAi技术在樱桃砧木中转化含有李属坏死环斑病毒（prunus necrotic ringspot virus, PNRSV）序列的hpRNA，在接穗中实现了对PNRSV的抗性[63-64]。amiRNA技术是将miRNA前体中的miRNA和miRNA*序列替换为相应的靶标序列，保留miRNA前体中的茎环结构，利用内源miRNA途径产生的小RNA沉默靶标基因。由于果树病毒产生vsiRNA的切点及切割效率等均不明确，amiRNA在果树抗病毒研究中的应用还有待于进一步研究。

3　展　望

果树中的病毒多为复合侵染，病毒种类多样，但我们对果树病毒的研究还远远不够，尤其对于一些引起症状很轻或者无症状的含量较低的病毒，但这些病毒会逐年累积，通过嫁接等途径不断传播，与其他病毒复合侵染可能加重病害。但受限于果树自身的特点和果树病毒研究手段的缺乏，其研究水平与模式植物及草本植物具有较大差距。

现阶段的研究已证实RNAi具有抗病毒作用，在病毒侵染的寄主植物细胞中产生大量vsiRNA，其装载到寄主AGO蛋白中以碱基互补配对的方式靶向病毒基因组。对拟南芥的遗传分析和多种植物vsiRNA表达谱的分析揭示了vsiRNA的起源和生物合成过程。虽然果树中与vsiRNA产生有关的内源RDR-DCL-AGO同源物的种类及其功能还未明确，有关小RNA介导的抗病毒基因沉默通路还有待于进一步解析，但基于vsiRNA起源和分布的特点，vsiRNA在果树病毒研究中已有广泛应用。由于果树病毒摩擦接种困难，显症周期长，症状表现不明显，且在不同品种上症状表现差异很大，果树病毒病原不易通过传统方法鉴定。因此，基于小RNA深度测序鉴定果树病毒病原的方法具有很大的发展前景。小RNA深度测序能够检测到已知的、未知的、含量高的及含量低的所有的RNA病毒、DNA病毒以及类病毒，在病毒病原鉴定、病毒检测及病毒种群研究中具有重要意义。虽然基于vsiRNA的抗病毒基因工程已有研究，但仍存在很多未知和挑战。由于果树病毒产生vsiRNA的切点和切割效率未知，不同病毒序列构成的hpRNA以及转化到不同寄主中产生的抗病毒效果可能不同。此外，对于vsiRNA介导的抗病毒沉默仍有很多未知内容：是否有大量的vsiRNA装载到特异的AGO蛋白中；即使一些AGO蛋白在病毒侵染过程中发挥作用且能结合vsiRNA，但是单独的RISC复合体由于其次级结构或病毒编码的RNA沉默抑制子活性可能并不靶向病毒基因组。目前，特异vsiRNA可以靶向病毒基因组从而抑制病毒侵染的体内实验证据还较少[65]。再者，果树中表达vsiRNA实现抗病毒需要转基因技术，而果树中高效的遗传转化方法也是果树研究的限制手段之一。因此，vsiRNA在果树抗病毒基因工程方面的应用还需要进一步的深入研究和实践。

参考文献 References：

[1] 张少瑜，张尊平 . 落叶果树病毒病及其研究进展[J]. 北方果树，2000(4): 1-4.
ZHANG Shaoyu, ZHANG Zunping. Virus disease of deciduous fruit trees and its research progress[J]. Northern Fruits, 2000(4): 1-4.

[2] 于云奇 . 小RNA深度测序在柑橘新发病毒的鉴定及TuMV诱导的抗病毒新机制中的研究[D]. 重庆: 西南大学，2017.
YU Yunqi. Researches of small RNA deep sequencing on the identification of new citrus viruses and novel antiviral silencing pathway triggered by TuMV infection[D]. Chongqing: Southwest University, 2017.

[3] CHEN L, YAN Z, XIA Z, CHENG Y, JIAO Z, SUN B, ZHOU T, FAN Z. A violaxanthin de-epoxidase interacts with a viral suppressor of RNA silencing to inhibit virus amplification[J]. Plant Physiology, 2017, 175(4): 1774-1794.

[4] ZHAO D, SONG G Q. Rootstock-to-scion transfer of transgene-derived small interfering RNAs and their effect on virus resistance in nontransgenic sweet cherry[J]. Plant Biotechnology Journal, 2014, 12(9): 1319-1328.

[5] WANG M B, METZLAFF M. RNA silencing and antiviral de-

fense in plants[J]. Current Opinion in Plant Biology, 2005, 8(2): 216-222.

[6] MOLNAR A, MELNYK C, BAULCOMBE D C. Silencing signals in plants: a long journey for small RNAs[J]. Genome Biology, 2011, 12(1): 215.

[7] DING S W, VOINNET O. Antiviral immunity directed by small RNAs[J]. Cell, 2007, 130(3): 413-426.

[8] BLEVINS T, RAJESWARAN R, AREGGER M, BORAH B K, SCHEPETILNIKOV M, BAERLOCHER L, FARINELLI L, MEINS F J R, HOHN T, POOGGIN M M. Massive production of small RNAs from a non-coding region of *Cauliflower mosaic virus* in plant defense and viral counter-defense[J]. Nucleic Acids Research, 2011, 39(12): 5003-5014.

[9] VANITHARANI R, CHELLAPPAN P, FAUQUET C M. Geminiviruses and RNA silencing[J]. Trends in Plant Science, 2005, 10(3): 144-151.

[10] DELERIS A, GALLEGO- BARTOLOME J, BAO J, KASSCHAU K D, CARRINGTON J C, VOINNET O. Hierarchical action and inhibition of plant dicer-like proteins in antiviral defense[J]. Science, 2006, 313(5783): 68-71.

[11] XIA Z, PENG J, LI Y, CHEN L, LI S, ZHOU T, FAN Z. Characterization of small interfering RNAs derived from *sugarcane mosaic virus* in infected maize plants by deep sequencing[J]. PLoS One, 2014, 9(5): e97013.

[12] XIA Z, ZHAO Z, CHEN L, LI M, ZHOU T, DENG C, ZHOU Q, FAN Z. Synergistic infection of two viruses MCMV and SCMV increases the accumulations of both MCMV and MCMV-derived siRNAs in maize [J]. Scientific Reports, 2016, 6(1): 20520.

[13] QIN C, LI B, FAN Y Y, ZHANG X, YU Z M, RYABOV E, ZHAO M, WANG H, SHI N N, ZHANG P C, JACKSON S, TOR M, CHENG Q, LIU Y L, GALLUSCI P, HONG Y G. Roles of dicer-like proteins 2 and 4 in intra- and intercellular antiviral silencing[J]. Plant Physiology, 2017, 174(2): 1067-1081.

[14] AKBERGENOV R, SI- AMMOUR A, BLEVINS T, AMIN I, KUTTER C, VANDERSCHUREN H, ZHANG P, GRUISSEM W, MEINS F J R, HOHN T, POOGGIN M M. Molecular characterization of geminivirus-derived small RNAs in different plant species[J]. Nucleic Acids Research, 2006, 34(2): 462-471.

[15] BLEVINS T, RAJESWARAN R, SHIVAPRASAD P V, BEKNAZARIANTS D, SI- AMMOUR A, PARK H S, VAZQUEZ F, ROBERTSON D, MEINS F J R, HOHN T, POOGGIN M M. Four plant dicers mediate viral small RNA biogenesis and DNA virus induced silencing[J]. Nucleic Acids Research, 2006, 34 (21): 6233-6246.

[16] MALLORY A, VAUCHERET H. Form, function, and regulation of ARGONAUTE proteins[J]. The Plant Cell, 2010, 22(12): 3879-3889.

[17] VOINNET O. Use, tolerance and avoidance of amplified RNA silencing by plants[J]. Trends in Plant Science, 2008, 13(7): 317-328.

[18] WANG X B, WU Q, ITO T, CILLO F, LI W X, CHEN X, YU J L, DING S W. RNAi-mediated viral immunity requires amplification of virus-derived siRNAs in *Arabidopsis thaliana*[J]. Proceedings of the National Academy of Sciences of the United States of America, 2010, 107(1): 484-489.

[19] MARTÍNEZ D E, ALBA A E, MORENO A B, GABRIEL M, MALLORY A C, CHRIST A, BOUNON R, BALZERGUE S, AUBOURG S, GAUTHERET D, CRESPI M D, VAUCHERET H, MAIZEL A. In plants, decapping prevents RDR6-dependent production of small interfering RNAs from endogenous mRNAs [J]. Nucleic Acids Research, 2015, 43(5): 2902-2913.

[20] VISSER M, MAREE H J, REES D J, BURGER J T. High-throughput sequencing reveals small RNAs involved in ASGV infection[J]. BMC Genomics, 2014, 15(1): 568.

[21] LIU J, ZHANG X J, YANG Y K, HONG N, WANG G P, WANG A M, WANG L P. Characterization of virus- derived small interfering RNAs in *Apple stem grooving virus*-infected in vitro-cultured *Pyrus pyrifolia* shoot tips in response to high temperature treatment[J]. Virology Journal, 2016, 13(1): 1-11.

[22] KREUZE J F, PEREZ A, UNTIVEROS M, QUISPE D, FUENTES S, BARKER I, SIMON R. Complete viral genome sequence and discovery of novel viruses by deep sequencing of small RNAs: a generic method for diagnosis, discovery and sequencing of viruses[J]. Virology, 2009, 388(1): 1-7.

[23] WU Q, DING S W, ZHANG Y, ZHU S. Identification of viruses and viroids by next-generation sequencing and homology-dependent and homology- independent algorithms[J]. Annual Review of Phytopathology, 2015, 53(1): 425-444.

[24] XIE L X, GAO F L, ZHENG S, ZHANG X Y, ZHANG L J, LI T. Molecular characterization of a new potyvirus infecting passion fruit[J]. Archives of Virology, 2019, 164(7): 1903-1906.

[25] 马宇欣，李世访 . 高通量测序技术在鉴定木本植物双生病毒中的应用[J]. 植物保护，2016，42(6): 1-10.
MA Yuxin, Li Shifang. Application of next-generation sequencing technology in identification of geminiviruses from woody plants[J]. Plant Protection, 2016, 42(6): 1-10.

[26] 苏秀 . 小 RNA 深度测序在几种木本植物病毒鉴定中的应用[D]. 杭州: 浙江大学，2015.
SU Xiu. Application of next-generation sequencing technology for identifying viruses from woody plants[D]. Hangzhou: Zhejiang University, 2015.

[27] RUIZ-RUIZ S, NAVARRO B, GISEL A, PENA L, NAVARRO L, MORENO P, DI SERIO F, FLORES R. Citrus tristeza virus infection induces the accumulation of viral small RNAs (21-24-nt) mapping preferentially at the 3’-terminal region of the genomic RNA and affects the host small RNA profile[J]. Plant Molecular Biology, 2011, 75(6): 607-619.

[28] SEGUIN J, RAJESWARAN R, MALPICA- LOPEZ N, MARTIN R R, KASSCHAU K, DOLJA V V, OTTEN P, FARINELLI L, POOGGIN M M. De novo reconstruction of consensus mas-

ter genomes of plant RNA and DNA viruses from siRNAs[J]. PLoS One, 2014, 9(2): e88513.

[29] VIVES M C, VELAZQUEZ K, PINA J A, MORENO P, GUERRI J, NAVARRO L. Identification of a new enamovirus associated with citrus vein enation disease by deep sequencing of small RNAs[J]. Phytopathology, 2013, 103(10): 1077-1086.

[30] ZHANG Z, QI S, TANG N, ZHANG X, CHEN S, ZHU P, MA L, CHENG J, XU Y, LU M, WANG H, DING S W, LI S, WU Q. Discovery of replicating circular RNAs by RNA-Seq and computational algorithms[J]. PLoS Pathogens, 2014, 10(12): e1004553.

[31] HE Y, CAI L, ZHOU L L, YANG Z K, HONG N, WANG G P, LI S F, XU W X. Deep sequencing reveals the first fabavirus infecting peach[J]. Scientific Reports, 2017, 7(1): 11329.

[32] ELBEAINO T, GIAMPETRUZZI A, DE STRADIS A, DIGIARO M. Deep-sequencing analysis of an apricot tree with vein clearing symptoms reveals the presence of a novel betaflexivirus[J]. Virus Research, 2014, 181: 1-5.

[33] LIANG P, NAVARRO B, ZHANG Z, WANG H, LU M, XIAO H, WU Q, ZHOU X, DI SERIO F, LI S. Identification and characterization of a novel geminivirus with a monopartite genome infecting apple trees[J]. Journal of General Virology, 2015, 96: 2411-2420.

[34] NODA H, YAMAGISHI N, YAEGASHI H, XING F, XIE J, LI S, ZHOU T, ITO T, YOSHIKAWA N. Apple necrotic mosaic virus, a novel ilarvirus from mosaic-diseased apple trees in Japan and China[J]. Journal of General Plant Pathology, 2017, 83(2): 83-90.

[35] SU X, FU S, QIAN Y, ZHANG L, XU Y, ZHOU X. Discovery and small RNA profile of *Pecan mosaic-associated virus*, a novel potyvirus of pecan trees[J]. Scientific Reports, 2016, 6: 26741.

[36] ZHENG Y, NAVARRO B, WANG G, WANG Y, YANG Z, XU W, ZHU C, WANG L, DI SERIO F, HONG N. *Actinidia chlorotic ringspot-associated virus*: a novel emaravirus infecting kiwifruit plants[J]. Molecular Plant Pathology, 2017, 18(4): 569-581.

[37] MARAIS A, FAURE C, MUSTAFAYEV E, BARONE M, ALIOTO D, CANDRESSE T. Characterization by deep sequencing of *Prunus* virus T, a novel *tepovirus* infecting *Prunus* species[J]. Phytopathology, 2015, 105(1): 135-140.

[38] 宗晓娟，王文文，魏海蓉，王甲威，陈新，徐丽，刘庆忠 . 3 种甜樱桃病毒 PNRSV、PDV 及 LChV-2 的多重 RT-PCR 检测方法的建立与应用[J]. 中国农业科学，2014，47(6): 1111-1118.
ZONG Xiaojuan, WANG Wenwen, WEI Hairong, WANG Jiawei, CHEN Xin, XU Li, LIU Qingzhong. Development and application of a multiplex RT-PCR assay for detecting three sweet cherry virus species[J]. Scientia Agricultura Sinica, 2014, 47(6): 1111-1118.

[39] 侯义龙，张开春，杨俊玲 . 应用 RT-PCR 方法检测桃和樱桃及其组培苗上的 PNRSV 和 PDV[J]. 果树学报，2005，22(3): 292-293.
HOU Yilong, ZHANG Kaichun, YANG Junling. Detection of PNRSV and PDV in peach and cherry by RT-PCR[J]. Journal of Fruit Science, 2005, 22(3): 292-293.

[40] 袁小环，李青，张开春 . 微茎尖培养脱除樱桃 2 种病毒试验[J]. 中国果树，2005(1): 40-42.
YUAN Xiaohuan, LI Qing, ZHANG Kaichun. Elimination of two cherry-infecting viurses by micro-stem culture[J]. China Fruits, 2005(1): 40-42.

[41] KUTNJAK D, RUPAR M, GUTIERREZ-AGUIRRE I, CURK T, KREUZE J F, RAVNIKAR M. Deep sequencing of virus-derived small interfering RNAs and RNA from viral particles shows highly similar mutational landscapes of a plant virus population[J]. Journal of Virology, 2015, 89(9): 4760-4769.

[42] STOBBE A H, ROOSSINCK M J. Plant virus metagenomics: what we know and why we need to know more[J]. Frontiers in Plant Science, 2014, 5: 150.

[43] BEERENWINKEL N, ZAGORDI O. Ultra-deep sequencing for the analysis of viral populations[J]. Current Opinion in Virology, 2011, 1(5): 413-418.

[44] LICCIARDELLO G, SCUDERI G, FERRARO R, GIAMPETRUZZI A, RUSSO M, LOMBARDO A, RASPAGLIESI D, BAR-JOSEPH M, CATARA A. Deep sequencing and analysis of small RNAs in sweet orange grafted on sour orange infected with two citrus tristeza virus isolates prevalent in Sicily[J]. Archives of Virology, 2015, 160(10): 2583-2589.

[45] ISAKOV O, BORDERIA A V, GOLAN D, HAMENAHEM A, CELNIKER G, YOFFE L, BLANC H, VIGNUZZI M, SHOMRON N. Deep sequencing analysis of viral infection and evolution allows rapid and detailed characterization of viral mutant spectrum[J]. Bioinformatics, 2015, 31(13): 2141-2150.

[46] VERBIST B, CLEMENT L, REUMERS J, THYS K, VAPIREV A, TALLOEN W, WETZELS Y, MEYS J, AERSSENS J, BIJNENS L, THAS O. ViVaMBC: estimating viral sequence variation in complex populations from illumina deep-sequencing data using model-based clustering[J]. BMC Bioinformatics, 2015, 16 (1): 59.

[47] CORRELL M, BAILEY A L, SARIKAYA A, O'CONNOR D H, GLEICHER M. LayerCake: a tool for the visual comparison of viral deep sequencing data[J]. Bioinformatics, 2015, 31(21): 3522-3528.

[48] CZOTTER N, MOLNAR J, PESTI R, DEMIAN E, BARATH D, VARGA T, VARALLYAY E. Use of siRNAs for diagnosis of viruses associated to woody plants in nurseries and stock collections[J]. Methods in Molecular Biology, 2018, 1746: 115-130.

[49] RAJAMAKI M L, LEMMETTY A, LAAMANEN J, ROININEN E, VISHWAKARMA A, STRENG J, LATVALA S, VALKONEN J P T. Small-RNA analysis of pre-basic mother plants and conserved accessions of plant genetic resources for the presence of viruses[J]. PLoS One, 2019, 14(8): e0220621.

[50] EICHMEIER A, KOMINKOVA M, PECENKA J, KOMINEK P.

High-throughput small RNA sequencing for evaluation of grapevine sanitation efficacy[J]. Journal of Virological Methods, 2019, 267: 66-70.

[51] VIGNE E, GARCIA S, KOMAR V, LEMAIRE O, HILY J M. Comparison of serological and molecular methods with high-throughput sequencing for the detection and quantification of grapevine fanleaf virus in vineyard samples[J]. Frontiers in Microbiology, 2018, 9: 2726.

[52] 彭向永，马锋旺，张开春 . 果树无病毒研究进展[J]. 西北农林科技大学学报（自然科学版），2003，31(6): 193-198.
PENG Xiangyong, MA Fengwang, ZHANG Kaichun. Progress of techniques for virus-free fruit trees[J]. Journal of Northwest Agriculture and Forestry University（Natural Science）, 2003, 31 (6): 193-198.

[53] MOCHIZUKI T, OHKI S T. Shoot meristem tissue of tobacco inoculated with *Cucumber mosaic virus* is infected with the virus and subsequently recovers from infection by RNA silencing[J]. Journal of General Plant Pathology, 2004, 70(6): 363-366.

[54] SUNPAPAO A, NAKAI T, DONG F, MOCHIZUKI T, OHKI S T. The 2b protein of cucumber mosaic virus is essential for viral infection of the shoot apical meristem and for efficient invasion of leaf primordia in infected tobacco plants[J]. Journal of General Virology, 2009, 90(12): 3015-3021.

[55] GUO Q, LIU Q, SMITH N A, LIANG G, WANG M B. RNA Silencing in plants: mechanisms, technologies and applications in horticultural crops[J]. Current Genomics, 2016, 17(6): 476-489.

[56] DUAN C G, WANG C H, GUO H S. Application of RNA silencing to plant disease resistance[J]. Silence, 2012, 3(1): 5.

[57] SMITH N A, SINGH S P, WANG M B, STOUTJESDIJK P A, GREEN A G, WATERHOUSE P M. Total silencing by intron-spliced hairpin RNAs[J]. Nature, 2000, 407(6802): 319-320.

[58] ILARDI V, NICOLA-NEGRI E D. Genetically engineered resistance to *Plum pox virus* infection in herbaceous and stone fruit hosts[J]. GM Crops, 2011, 2(1): 24-33.

[59] BEACHY R N. Mechanisms and applications of pathogen-derived resistance in transgenic plants[J]. Current Opinion in Biotechnology , 1997, 8(2): 215-220.

[60] HILY J M, SCORZA R, WEBB K, RAVELONANDRO M. Accumulation of the long class of siRNA is associated with resistance to *Plum pox virus* in a transgenic woody perennial plum tree[J]. Molecular Plant-Microbe Interactions, 2005, 18(8): 794-799.

[61] BROSNAN C A, VOINNET O. Cell-to-cell and long-distance siRNA movement in plants: mechanisms and biological implications[J]. Current Opinion in Plant Biology, 2011, 14(5): 580-587.

[62] LEMGO G N Y, SABBADINI S, PANDOLFINI T, MEZZETTI B. Biosafety considerations of RNAi-mediated virus resistance in fruit-tree cultivars and in rootstock[J]. Transgenic Research, 2013, 22(6): 1073-1088.

[63] SONG G Q, SINK K C, WALWORTH A E, COOK M A, ALLISON R F, LANG G A. Engineering cherry rootstocks with resistance to *Prunus necrotic ring spot virus* through RNAi-mediated silencing[J]. Plant Biotechnology Journal, 2013, 11(6): 702-708.

[64] ZHAO D, SONG G Q. Rootstock-to-scion transfer of transgene-derived small interfering RNAs and their effect on virus resistance in nontransgenic sweet cherry[J]. Plant Biotechnology Journal, 2014, 12(9): 1319-1328.

[65] ZHANG C, WU Z, LI Y, WU J. Biogenesis, function, and applications of virus-derived small RNAs in plants[J]. Frontiers in Microbiology, 2015, 6: 1237.