Trends in Plant Science

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Opinion

Minimal small RNA precursors unlock virus-based precision RNAi

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Artificial small RNAs (art-sRNAs) mediate highly specific RNAi in plants, but their broader use has been constrained by long precursor architectures and dependence on transgenic delivery. Recent work shows that accurate and efficient art-sRNA biogenesis can be achieved using precursors of minimal size rather than full-length endogenous scaffolds, without compromising silencing efficacy. We discuss how minimal art-sRNA precursors represent a versatile platform for precision RNAi and, critically, unlock RNA viruses as stable, programmable platforms for the production of highly specific art-sRNAs. In other words, minimal art-sRNA precursors transform RNA viruses from generators of heterogeneous small RNA populations into nontransgenic, programmable vectors that deliver defined art-sRNAs for precision gene silencing and antiviral protection.

Highlights

Artificial small RNA precursors can be drastically shortened without compromising processing accuracy or silencing efficacy.

Minimal precursors expressed from RNA viruses produce authentic artificial small RNAs, including both artificial microRNAs and synthetic trans-acting small interfering RNAs.

Minimal precursors expressed from RNA viruses produce authentic artificial microRNAs by co-opting cytoplasmic antiviral RNAi pathways.

Viral vectors expressing artificial small RNAs support efficient silencing of endogenous genes.

Viral vectors expressing synthetic trans-acting small interfering RNAs can function as effective antiviral vaccines for plant protection.

Viral vectors expressing artificial small RNAs can be applied transgene-free by spraying crude extracts obtained from infected plants.

Towards next-generation precision silencing: the rise of artificial small RNAs

RNA interference (RNAi) (see Glossary) lies at the core of eukaryotic gene regulation and antiviral defense [1,2]. In plants, this conserved pathway relies on DICER-LIKE (DCL) enzymes to process double-stranded RNA (dsRNA) into small RNAs (sRNAs), which are then loaded into ARGONAUTE (AGO) proteins to form RNA-induced silencing complexes (RISCs). Guided by sequence complementarity, RISCs repress target transcripts with high efficiency. This regulatory landscape is shaped by three major sRNA classes: microRNAs (miRNAs), small interfering RNAs (siRNAs), and **phased siRNAs (phasiRNAs)**, a subset of which acts *in trans* as trans-acting siRNAs (tasiRNAs), collectively tuning development, stress responses, and genome stability [3].

For decades, classic RNAi technologies have exploited this endogenous machinery using long double-stranded RNA (dsRNA) triggers. **Virus-induced gene silencing (VIGS)** delivers several hundred nucleotide (nt) target fragments through viral genomes, while hairpin RNA transgenes express inverted repeats that generate dsRNA [4,5]. Although powerful, these first-generation strategies share a fundamental limitation: long dsRNAs are processed by multiple DCLs (notably DCL2, DCL3, and DCL4), generating large and heterogeneous populations of siRNAs with variable sizes and sequences. This 'shotgun' output increases the likelihood of **off-target effects** and complicates both phenotypic interpretation and practical use [6]. miRNA-induced gene silencing (MIGS) partially mitigated this issue by routing dsRNA processing predominantly through DCL4 to generate phased siRNAs [7,8], but these siRNAs lack sequence-validated specificity for their intended target(s).

The demand for higher specificity has led to a shift toward second-generation RNAi strategies based on **artificial small RNAs** (art-sRNAs), including **artificial microRNAs** (amiRNAs) and **synthetic trans-acting siRNAs** (syn-tasiRNAs) [9] (Box 1). These molecules are computationally designed to maximize on-target activity while excluding unintended transcripts [20,21] and are embedded within defined precursor architectures that control their biogenesis. As a result, art-sRNAs replace bulk dsRNA processing with the programmed production of predefined

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21-nt guides, transforming RNAi from a probabilistic outcome into a predictable and programmable process.

Minimal art-sRNA precursors: a versatile platform for precise RNA silencing

Early art-sRNA platforms were built on full-length endogenous precursors. AmiRNAs were commonly embedded in native primary *MIRNA* (*pri-MIRNA*) transcripts of several hundred nts (e.g., *MIR319* and *MIR390a*), while syn-tasiRNAs were produced from modified *TAS* transcripts (e.g., *TAS1c* and *TAS3*) that often exceeded 1 kb [10,22,23]. These designs retained all recognizable structural features (basal stems, distal stem-loops, spacers, and flanking sequences) on the assumption that length and redundancy were required for robust and accurate processing.

A recent body of evidence now challenges this assumption. Across both amiRNA and syn-tasiRNA systems, accurate and efficient silencing depends not on precursor complexity but on a limited set of biogenesis determinants that can be retained in dramatically shortened scaffolds. This reframes precursor engineering as an optimization problem: identifying the **minimal precursor** architecture that preserves accurate processing and full silencing efficacy.

For amiRNAs, systematic truncation of the widely used *Arabidopsis thaliana MIR390a* precursor revealed that accurate DCL1-dependent processing can be achieved with a chimeric scaffold of only 89 nt [15]. This shortened chimeric (***shc***) precursor retains the complete *MIR390a* basal stem but removes dispensable flanking regions and replaces the distal stem-loop with a compact 16-nt element from *Oryza sativa MIR390* carrying a 2-nt deletion (Figure 1) [15]. Despite its reduced size, *shc* supports accurate amiRNA biogenesis and robust silencing of endogenous genes in *A. thaliana* and *Nicotiana benthamiana* [15], highlighting that only a subset of *pri-MIRNA* structural features is functionally required.

An analogous principle applies to syn-tasiRNAs. Minimal syn-tasiRNA precursors retain only the elements actively used by the tasiRNA pathway: a 22-nt miRNA target site to initiate processing, a short spacer to define register, and one or more phased 21-nt syn-tasiRNA units (Figure 1). These compact cassettes, as short as 54 nt for a single guide, produce authentic, accurately phased syn-tasiRNAs with efficiencies comparable to full-length *TAS* transcripts [24]. Silencing

Box 1. Art-sRNA biogenesis pathways

Art-sRNAs are engineered 21-nt guides that exploit endogenous RNAi pathways to silence target RNA with high specificity. In plants, the two main art-sRNA classes, amiRNAs and syn-tasiRNAs, differ in precursor architecture and biogenesis route but converge on AGO-mediated target cleavage (Figure I). Art-sRNA pathways are initiated when a transcript presents a minimal set of structural and sequence determinants sufficient to engage the host RNAi machinery. Importantly, both pathways can be initiated by either full-length endogenous scaffolds or minimal synthetic precursors, provided that essential recognition and processing determinants are preserved.

AmiRNAs are produced from modified *MIRNA* precursors in which the endogenous miRNA/miRNA star(*) duplex is replaced by a designed amiRNA/amiRNA* pair [10]. The *aMIRNA* transgene is transcribed in the nucleus, processed by DCL1 into a 21-nt duplex [11], which is subsequently methylated by HEN1 [12,13]. The guide strand, typically with a 5′ terminal uracil (U), is then loaded into AGO1 to cleave complementary mRNAs, as occurs with endogenous miRNAs [14]. Recent work shows that amiRNA biogenesis can be supported by minimal precursors such as the 89-nt *shc* scaffold [15].

Syn-tasiRNAs are derived from trans-acting siRNA-generating (*TAS*)-like transcripts and enable multiplex targeting from a single precursor. The pathway can be initiated either from full-length *TAS* scaffolds or from minimal syn-tasiRNA precursors that retain only the essential elements: a 22-nt miRNA trigger site, a short spacer, and one or more 21-nt syn-tasiRNA units with a 5′ terminal U for AGO1 association. In both cases, biogenesis is typically initiated by cleavage of the precursor by a 22-nt miRNA–AGO complex [16,17], followed by stabilization of the cleaved RNA by SGS3, conversion into dsRNA by RDR6, and phased processing by DCL4 to produce one or more 21-nt syn-tasiRNAs, as in endogenous tasiRNA pathways [18,19]. When multiple syn-tasiRNAs are generated, they can simultaneously target multiple sites within a single transcript or distinct RNAs.

Glossary

AmiR-VIGS: a class of virus-induced gene silencing that uses a viral vector to produce an artificial microRNA.
Art-sRNA-VIGS: a class of virus-induced gene silencing that uses a viral vector to produce one or more artificial small RNAs.
Artificial microRNA (amiRNA): computationally designed small RNA produced *in planta* from an endogenous or modified *MIRNA* precursor, engineered to target complementary RNA with high specificity. Although most amiRNAs are designed as 21-nt guides for high specificity, 22-nt amiRNAs can also be generated and may promote secondary siRNA production.
Artificial small RNA (art-sRNA): computationally designed small RNA, typically 21 nts in length, produced *in planta* from an endogenous or modified precursor and engineered to target complementary RNA with high specificity. In precision RNAi applications, 21-nt designs are generally preferred because they minimize secondary siRNA production and help preserve target specificity.
Crude extracts: a liquid preparation obtained by mechanically disrupting biological tissue in a stabilizing phosphate buffer, containing a mixture of macromolecules and particles. Crude extracts prepared from virus-infected plant tissue are used to transmit viral vectors and induce artificial small RNA-mediated gene silencing without DNA delivery.
Minimal precursor: an engineered RNA precursor reduced to the essential sequence required to maintain the structural features for correct processing and high silencing efficiency, typically ensuring stability in viral vectors, where full-length precursors are often unstable.
Off-target effects: unintended gene-silencing events occurring when small RNAs share partial sequence complementarity with cellular transcripts other than the specific target.
Phased small interfering RNA (phasiRNA): 21-nt secondary small interfering RNA produced in a defined register from a precursor transcript after trigger-mediated cleavage, usually involving RDR6-dependent dsRNA synthesis and DCL processing. PhasiRNAs may act *in cis* or *in trans*, but *trans*-acting activity requires experimental or predicted target complementarity.

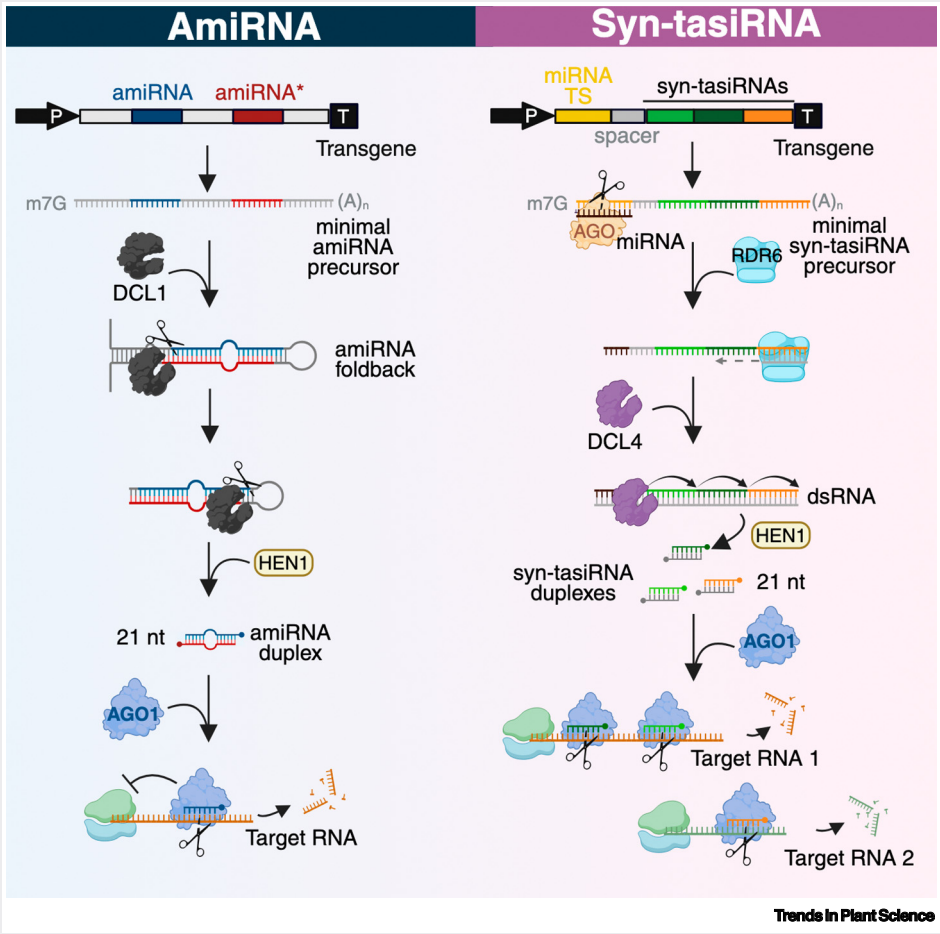

Figure I. Biogenesis pathways of art-sRNAs expressed from transgenes in plants. Artificial microRNAs (amiRNAs) and synthetic trans-acting siRNAs (syn-tasiRNAs) are shown as derived from minimal precursors expressed from transgenes (P: promoter; T: terminator), although both pathways can also be initiated from full-length endogenous scaffolds. AmiRNAs are processed by DICER-LIKE 1 (DCL1) to produce a single 21-nucleotide (nt) guide loaded into ARGONAUTE 1 (AGO1), whereas syn-tasiRNAs are produced through miRNA-triggered cleavage, RNA-dependent RNA polymerase 6 (RDR6) amplification, and phased DCL4 processing, generating one or multiple 21-nt guides. Both pathways converge on AGO1-mediated target cleavage or translational repression of target RNA(s). Figure created using BioRender (https://BioRender.com/dojerk0).

RNA interference (RNAi): a conserved eukaryotic gene-silencing mechanism in which small RNAs direct the sequence-specific repression of complementary messenger RNAs or genomic regions.
Shortened chimeric (*shc*): artificial microRNA precursor derived from *MIR390* scaffolds. This 89-nt precursor preserves the full *A. thaliana MIR390a* basal stem, removes nonessential flanking RNA, and swaps the distal stem-loop for a 16-nt *O. sativa MIR390*-derived sequence carrying a 2-nt deletion.
Synthetic trans-acting small interfering RNA (syn-tasiRNA): computationally designed 21-nt phased small interfering RNA, produced from an endogenous *TAS* precursor or an engineered phasiRNA-generating transcript, and designed to target complementary RNA with high specificity.
Syn-tasiR-VIGS: a class of virus-induced gene silencing that uses a viral vector to produce one or more synthetic trans-acting small interfering RNAs.
Virus-induced gene silencing (VIGS): a transient reverse-genetics technique that uses a viral vector carrying a target gene fragment to produce a heterogeneous population of small interfering RNAs.

performance instead depends on trigger compatibility and availability. Minimal syn-tasiRNA precursors require an appropriate 22-nt miRNA in the host species, so their processing depends on the abundance and spatiotemporal expression of that trigger. This dependency may limit syn-tasiRNA production in some tissues, stages, or conditions, but can also be exploited: endogenous miRNA expression maps could help predict syn-tasiRNA production and guide trigger selection for specific crops, tissues, or applications. Compatible triggers have already enabled clear late-flowering and chlorophyll-associated bleaching phenotypes in model and crop plants [24,25].

Whether routed through endogenous miRNA or tasiRNA pathways, efficient silencing depends on the retention of essential processing determinants required for accurate processing, rather than on full-length precursor complexity. Importantly, this reduction is not merely neutral but

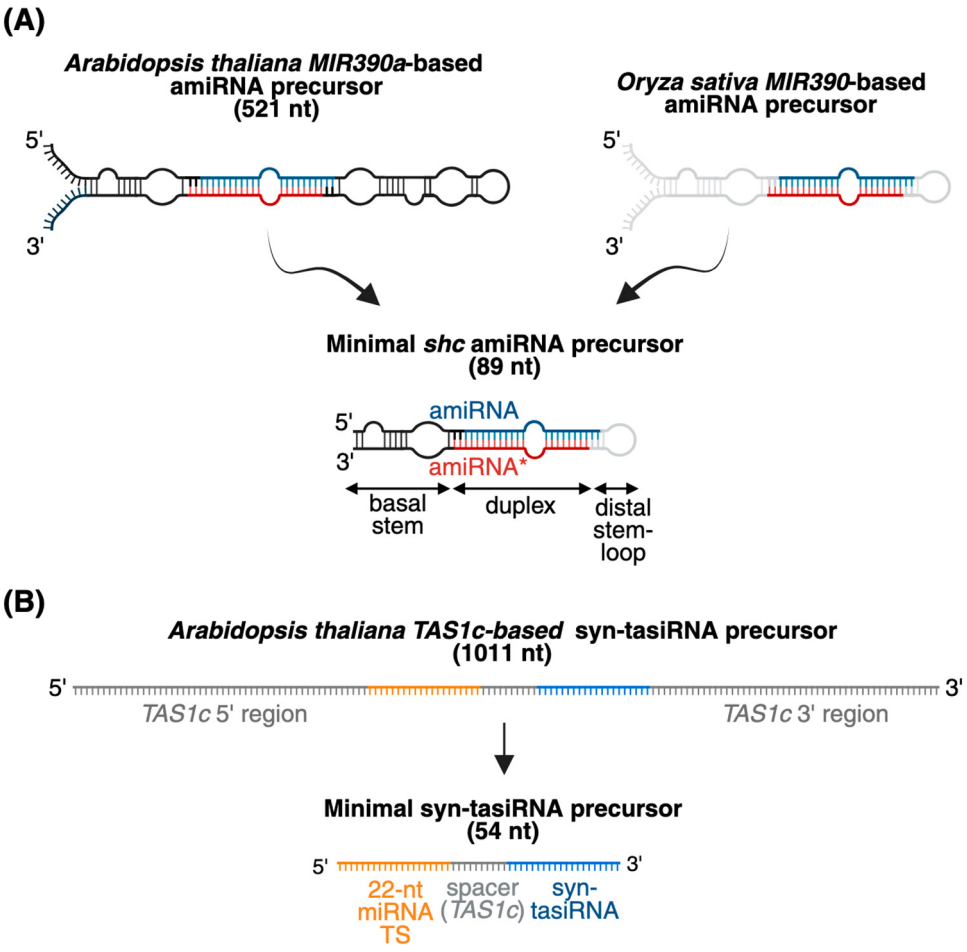

Figure 1. Structure of full-length and minimal amiRNA or syn-tasiRNA precursors. (A) Artificial microRNA (amiRNA) precursors. Full-length *MIR390* precursors from *A. thaliana* and *O. sativa* (rice) are reduced to an 89-nucleotide minimal amiRNA scaffold comprising the *A. thaliana MIR390a* basal stem, the engineered amiRNA/amiRNA* duplex, and a shortened distal stem-loop derived from rice *MIR390* with a 2-nucleotide (nt) deletion. (B) Synthetic trans-acting small interfering RNA (syn-tasiRNA) precursors. Full-length *A. thaliana TAS1c* precursor is reduced to a minimal syn-tasiRNA precursor of 54 nt for a single guide, containing only a 22-nt endogenous miRNA target site (TS), an 11-nucleotide spacer, and the syn-tasiRNA unit(s). *shc: shortened chimeric.* Figure created using BioRender (https://BioRender.com/yitlly).

advantageous: compact precursors simplify cloning and multiplexing, reduce synthesis costs, and, most critically, allow stable expression from delivery systems with strict cargo constraints.

Art-sRNA-VIGS: virus-delivered art-sRNAs for systemic gene silencing and antiviral vaccination

The reduction of art-sRNA precursors to their minimal functional architecture fundamentally changes how RNA viruses can be used for plant RNAi. Long inserts derived from endogenous *MIRNA* or *TAS* loci are intrinsically unstable in RNA viral genomes and are rapidly lost during replication [15,24]. In contrast, minimal amiRNA and syn-tasiRNA precursors are compact enough to be stably maintained, allowing viruses to function as reliable platforms for art-sRNA production. These approaches are collectively referred to as **Art-sRNA-VIGS**, including amiRNA-based VIGS (**AmiR-VIGS**) and syn-tasiRNA-based VIGS (**Syn-tasiR-VIGS**) (Figure 2).

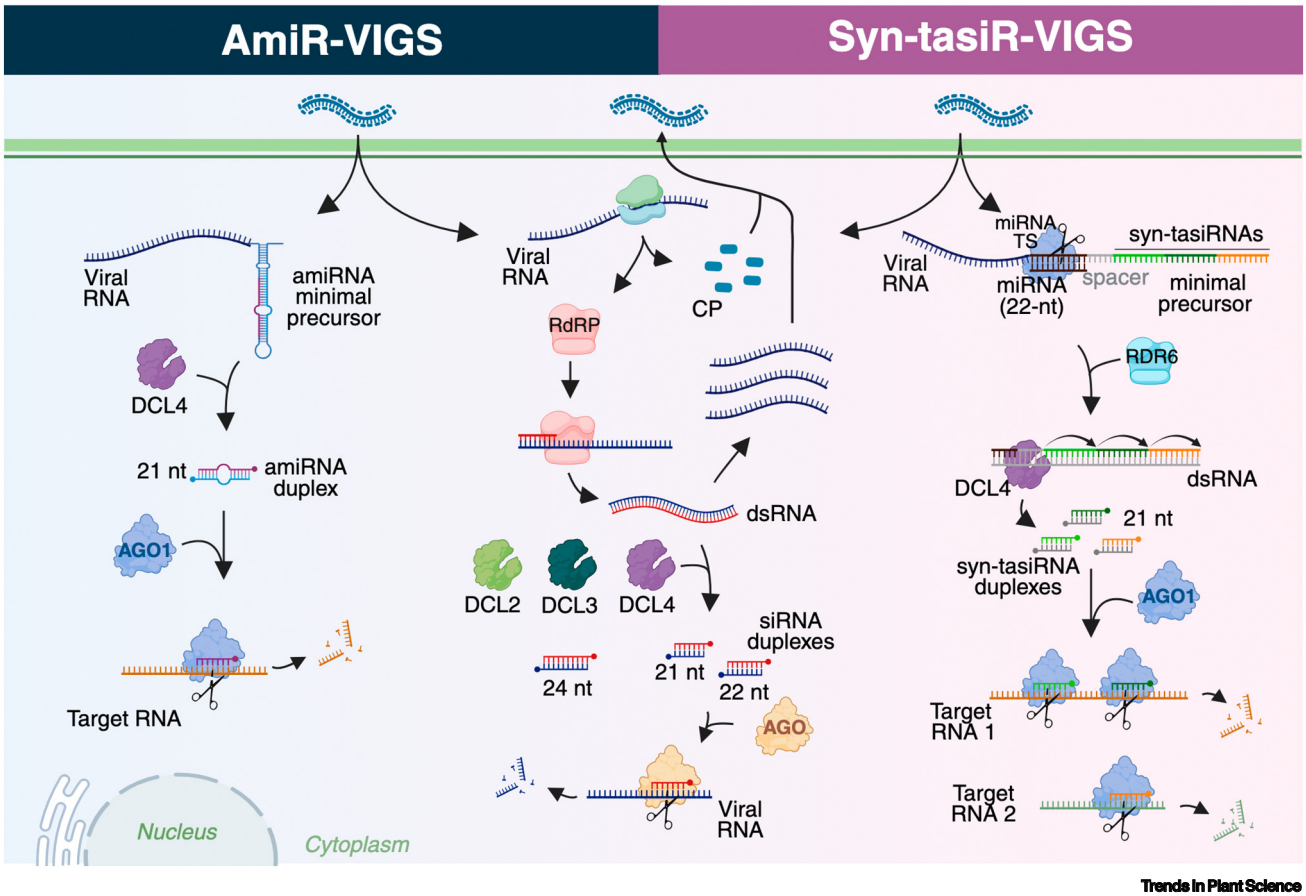

Figure 2. Model for amiR-VIGS and syn-tasiR-VIGS in plants. Minimal artificial small RNA (art-sRNA) precursors are preferentially and accurately processed within the highly competitive environment of viral replication, enabling precise gene silencing without transitivity or loss of viral vector propagation. Left: amiR-VIGS. Upon infection, a subset of viral RNAs carrying minimal amiRNA precursors enters a cytoplasmic RNAi pathway, where structured transcripts are processed by DICER-LIKE 4 (DCL4) into defined amiRNA duplexes that load into ARGONAUTE 1 (AGO1) and direct target mRNA cleavage, while other viral RNAs support replication and systemic spread. Middle: antiviral RNAi during viral replication. Viral RNA-dependent RNA polymerase (RdRP) generates double-stranded RNA (dsRNA) replication intermediates, which are processed by DCL2, DCL3, and DCL4 into heterogeneous antiviral siRNAs incorporated into AGO (e.g., AGO1 and AGO2) and targeting viral RNAs. Right: syn-tasiR-VIGS. Viral RNAs encoding minimal syn-tasiRNA precursors are cleaved by a 22-nt miRNA–AGO complex, triggering RNA-dependent RNA polymerase 6 (RDR6)-dependent dsRNA synthesis and phased DCL4 processing into defined syn-tasiRNA duplexes that load into AGO and mediate highly specific cleavage of one or multiple target mRNAs. As in amiR-VIGS, viral RNAs are partitioned between art-sRNA biogenesis and viral replication/movement. AmiR-VIGS: amiRNA-based VIGS; CP: coat protein; syn-tasiRNA: synthetic trans-acting siRNAs; syn-tasiR-VIGS: syn-tasiRNA-based VIGS; TS: target site. Figure created using BioRender (https://BioRender.com/ojf9ucn).

A key requirement for precise art-sRNA-VIGS is that art-sRNA biogenesis remains accurate during viral replication, preserving the production of defined guide RNAs. Viral RNAs are simultaneously subject to antiviral RNAi defense pathways that convert them into dsRNA substrates for DCL2, DCL3, and DCL4, producing heterogeneous populations of siRNAs, which form the mechanistic basis of classical VIGS [26]. If these pathways dominated, art-sRNA precursors would be fragmented into diverse siRNAs, precluding the accumulation of functional guides.

Empirical analyses across multiple virus–host combinations show that this is not the case. When expressed from RNA viruses, minimal art-sRNA precursors are generally processed with high accuracy, yielding the expected 21-nt amiRNAs or syn-tasiRNAs as the dominant species, with only minor accumulation of alternative precursor-derived sRNAs, as shown in both model and crop plants using distinct RNA-based viral vectors [15,24,25,27]. Only when up to four syn-tasiRNAs are encoded within a single minimal precursor does the accumulation of the third

and fourth guides decrease [24,27], suggesting a quantitative limit to multiplexing rather than a loss of processing accuracy. Importantly, secondary phased siRNAs from target transcripts are not detected, indicating that art-sRNA-VIGS does not trigger transitivity, reinforcing its specificity [15,27]. Collectively, these observations demonstrate that minimal precursors are preferentially and robustly processed even within the highly competitive environment of viral replication.

This high processing precision observed during art-sRNA-VIGS is striking, given that art-sRNA biogenesis occurs in the context of viral replication and active antiviral RNAi. For syn-tasiRNAs, this precision is readily explained: all required components of tasiRNA biogenesis [including miRNA-guided cleavage, RNA-dependent RNA polymerase 6 (RDR6)-mediated amplification, and DCL4 processing] operate in the cytoplasm [18], where viral RNAs replicate. In contrast, accurate amiRNA production from viral transcripts is more unexpected, as canonical miRNA precursors are normally processed in the nucleus by DCL1 [11]. Genetic analyses resolve this apparent discrepancy by showing that viral amiRNA biogenesis bypasses nuclear DCL1 and instead depends on DCL4 [15]. This is consistent with previous work showing that DCL4, the primary DCL in antiviral defense [28,29], can recognize and accurately process structured single-stranded RNAs resembling pre-miRNA or tasiRNA substrates [30]. Together, these findings indicate that viral art-sRNA precursors are routed into a cytoplasmic processing pathway in which DCL4 acts as a central hub, supporting accurate amiRNA and syn-tasiRNA production despite intense antiviral silencing pressure.

Transgene-free art-sRNA-VIGS: from functional genomics to next-generation antiviral vaccines

A long-standing constraint of plant RNAi technologies has been their reliance on DNA delivery, either through stable transformation or transient expression systems. Even classical VIGS, often described as transient or nontransgenic, still depends on recombinant DNA to initiate infection, limiting scalability, regulatory acceptance, and field deployment [26]. Transgene-free art-sRNA-VIGS, therefore, represents not a marginal improvement but a qualitative shift in how gene silencing can be applied in plant research and crop protection [31]. Still, viral delivery also entails inherent VIGS-related constraints. Broader application will require vectors with a suitable host range, efficient infection, low symptomatology, stable insert retention, and compatibility with crop-management practices. Because this approach still relies on infectious viral material, biosafety and regulatory aspects, including containment, nontarget spread, recombination, and persistence, must be evaluated case by case for each virus–host system.

Minimal art-sRNA precursors enable this shift. Their compact size and genetic stability allow viral vectors to be propagated in donor plants and subsequently applied through the spraying of **crude extracts** derived from non-agroinfiltrated tissues, avoiding transgenesis while allowing straightforward scaling and large-area application [15,24,25,27] (Box 2). This strategy critically

Box 2. Transgene-free art-sRNA-VIGS

VIGS is often considered nontransgenic, yet it still relies on recombinant DNA to initiate infection, limiting its scalability and broader biotechnological use [26]. Transgene-free art-sRNA-VIGS overcomes this limitation. Viral vectors encoding minimal art-sRNA precursors are first propagated in donor *N. benthamiana* plants by agroinoculation. Infected apical tissue is harvested and mechanically homogenized in a simple phosphate-based buffer, clarified by filtration, and supplemented with silicon carbide as a mild abrasive. The resulting extracts are applied directly to young plants by spray inoculation [15,24,25,27], a scalable method that allows efficient viral entry without specialized equipment (Figure I).

Following application, viral RNA replication and art-sRNA biogenesis occur entirely in the cytoplasm, where minimal precursors are processed with high accuracy to produce single, predefined 21-nt guides. Despite operating under active antiviral RNA silencing, this approach supports robust and specific silencing of endogenous genes and allows programmable antiviral vaccination using syn-tasiRNAs (Figure I), in which multiple guides from a single precursor simultaneously target distinct viral sites, reducing the likelihood of viral escape [15,24,25,27].

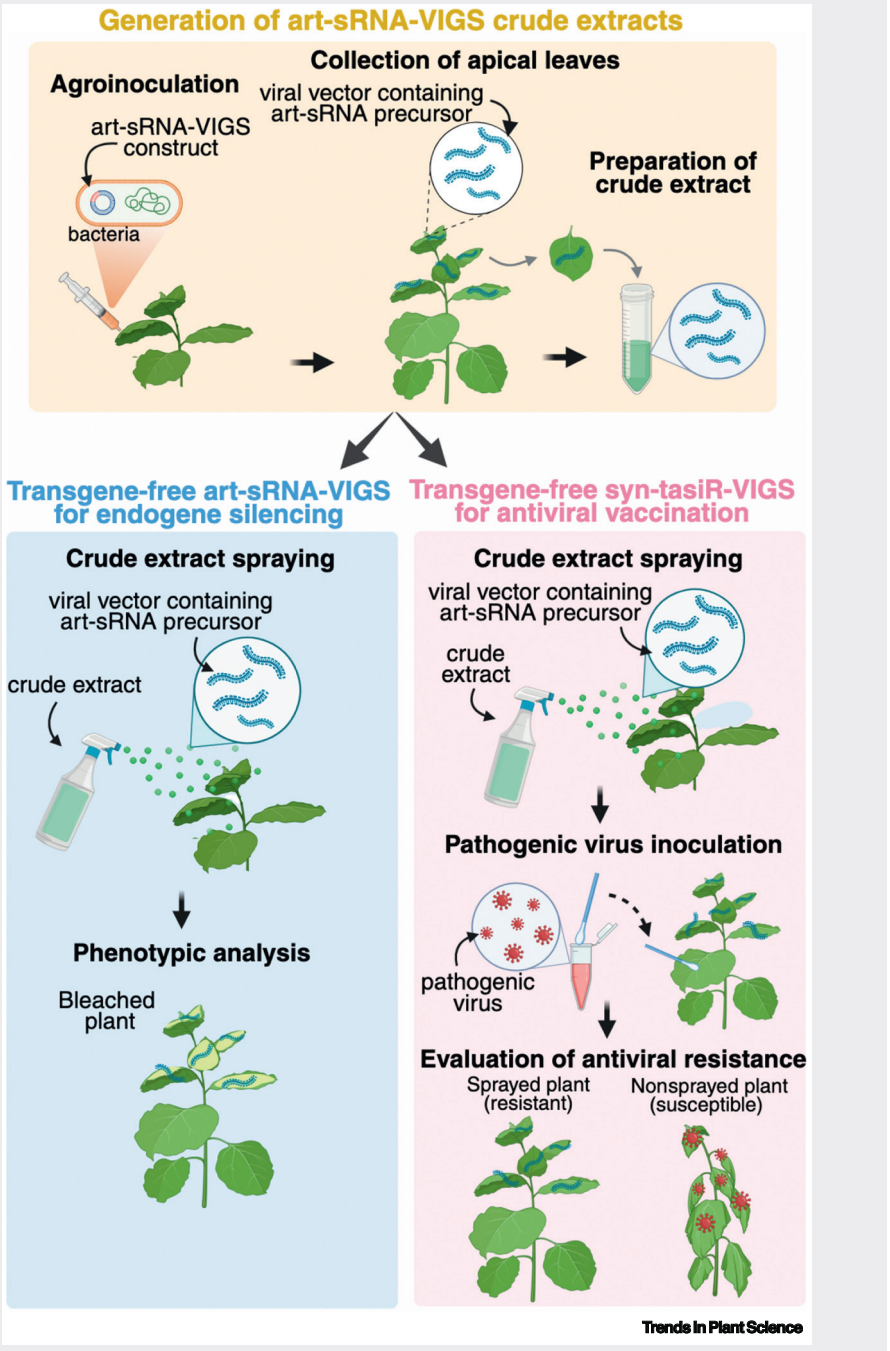

Figure I. Transgene-free art-sRNA-VIGS. Left: generation of artificial small RNA (art-sRNA) crude extracts. Viral vectors carrying minimal art-sRNA precursors are amplified in donor plants, from which apical tissue is collected to prepare infectious crude extracts containing the viral vector and art-sRNA precursor. Right: these extracts are applied to recipient plants by spray inoculation, enabling DNA-free delivery of viral RNA. Top: art-sRNA-based virus-induced gene silencing (art-sRNA-VIGS) for silencing endogenous genes, illustrated by a visible loss-of-function bleaching phenotype. Bottom: syn-tasiRNA-based VIGS (syn-tasiR-VIGS) for antiviral vaccination, where plants treated with syn-tasiRNA-containing extracts acquire resistance to subsequent infection by a pathogenic virus. Figure created using BioRender (https://BioRender.com/mhtt0hv).

depends on the genetic stability of minimal precursor inserts, which remain stable in RNA viral genomes despite strong recombination pressure.

Functionally, transgene-free art-sRNA-VIGS supports efficient silencing of endogenous genes across species [15,24,25,27]. Crude-extract application induces clear systemic silencing phenotypes that mirror those obtained through conventional agroinfiltration-based VIGS, while maintaining the hallmark features of art-sRNA technologies: defined 21-nt guides, absence of secondary siRNA amplification, and high target specificity. In this context, RNAi is no longer coupled to DNA constructs or agroinfiltration-based delivery but becomes a portable and scalable tool.

Beyond functional genomics, transgene-free syn-tasiR-VIGS extends to antiviral protection. Unlike classical cross-protection, which relies on mild viral strains and is typically limited to closely related viruses [32], syn-tasiR-VIGS enables sequence-defined, programmable antiviral vaccines that can be designed against one or more target viruses. By expressing multiple syn-tasiRNAs from a single precursor, a small, validated set can be packaged into one viral vector and delivered in a single nontransgenic dose, providing multitarget antiviral protection that limits viral escape by mutation [24,25,27,33].

Taken together, transgene-free amiR-VIGS and syn-tasiR-VIGS redefine the scope of RNAi in plants. By combining minimal precursor design with viral systemic mobility, these approaches transform RNAi from a research-limited genetic tool into a programmable, nontransgenic technology for gene regulation and plant health management.

Concluding remarks and future perspectives

Recent work shows that accurate and efficient art-sRNA biogenesis can be achieved using precursors of minimal size rather than full-length endogenous scaffolds, without compromising silencing efficacy. By reducing precursor architecture to its functional core, these designs consistently yield single, predefined 21-nt guides, avoid secondary siRNA amplification, and deliver reproducible silencing across species. Importantly, minimizing the size of the art-sRNA precursors has consequences that extend beyond molecular precision. Compact art-sRNA cassettes are preferentially retained in RNA viral genomes and are generally processed with high accuracy even under strong antiviral pressure, allowing stable viral delivery. This property repositions RNA viruses from passive generators of diverse siRNAs into programmable, nontransgenic vectors for defined gene regulation and antiviral protection. In contrast, other short VIGS inserts rely on heterogeneous pools of 21- and 22-nt siRNAs at the target locus [34], a mode of action likely promoting transitivity and thus compromising specificity.

Looking ahead, a key challenge is extending art-sRNA-VIGS to a broader range of crops while preserving robustness, specificity, and scalability. Priorities include expanding viral vector repertoires with defined host ranges, implementing attenuation strategies that balance systemic spread with minimal symptoms, and further optimizing syn-tasiR-VIGS through improved trigger availability or self-contained trigger-precursor designs. Notably, existing regulatory frameworks for natural viruses may ease the path toward acceptance of recombinant viral platforms [35]. In parallel, minimal precursor designs can be extended to virus-independent, transgene-free RNAi strategies, including topical RNA delivery [36–38], nanocarrier-mediated uptake [39,40], and expression from edited endogenous sRNA loci [41,42].

In any case, the recent body of work discussed in this opinion article supports an optimistic outlook in which minimal art-sRNA precursors emerge as a versatile and extensible platform for

Outstanding questions

What defines the minimal information content required for accurate artificial small RNA precursor processing? Which elements (cleavage register, spacer length, and local secondary structure) are truly indispensable, and which are evolutionary relics?

How do minimal precursors maintain processing accuracy under intense antiviral defense? Why are they protected from DICER-LIKE promiscuity and RNA-dependent RNA polymerase amplification, and does this reflect an unappreciated hierarchy in RNAi substrate selection?

How does the spatiotemporal expression of endogenous 22-nucleotide miRNAs constrain or allow synthetic trans-acting siRNAs biogenesis *in vivo*? Can endogenous miRNA expression maps be exploited to predict where and when synthetic trans-acting siRNAs will be produced?

What limits multiplexing from minimal artificial small RNA precursors during viral delivery, and can it be engineered? Viral expression preserves accurate phasing, yet accumulation of later guides drops beyond two to four synthetic trans-acting siRNAs. What defines the number of guides produced *in vivo*, and can redesigned architectures overcome it?

What are the real-world constraints on transgene-free artificial small RNA-based virus-induced gene-silencing delivery across species and environments? How do extract preparation, storage, freezing, and transport affect viral infectivity and silencing reproducibility?

Can synthetic trans-acting siRNAs-based virus-induced gene-silencing vaccines cure plants that are already infected, rather than only protecting against future infection? Can synthetic trans-acting siRNAs-based virus-induced gene silencing reduce viral load once infection is established? What constraints (e.g., viral replication dynamics, tissue accessibility, or competition between pathogenic and vaccine viruses) set the boundary between protection and cure?

precision RNAi that should extend beyond antiviral protection to the programmable control of pests, fungi, and bacteria through cross-kingdom [43,44] or within-plant RNA trafficking [45] (see Outstanding questions).

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Declaration of interests

A.E.C. and A.C. are inventors on patent applications related to minimal microRNA precursors (WO2025/026950) and minimal syn-tasiRNA precursors (WO2025/026951), owned by Agencia Estatal Consejo Superior de Investigaciones Científicas (CSIC).

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT 5.2 in order to improve the text and summarize content. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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