Hammerhead Ribozymes Against Virus and Viroid RNAs

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Abstract The hammerhead ribozyme, a small catalytic motif that promotes self-cleavage of the RNAs in which it is found naturally embedded, can be manipulated to recognize and cleave specifically in *trans* other RNAs in the presence of Mg^{2+}. To be really effective, hammerheads need to operate at the low concentration of Mg^{2+} existing in vivo. Evidence has been gathered along the last years showing that tertiary stabilizing motifs (TSMs), particularly interactions between peripheral loops, are critical for the catalytic activity of hammerheads at physiological levels of Mg^{2+}. These TSMs, in two alternative formats, have been incorporated into a

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new generation of more efficient *trans*-cleaving hammerheads, some of which are active in vitro and *in planta* when targeted against the highly structured RNA of a viroid (a small plant pathogen). This strategy has potential to confer protection against other RNA replicons, like RNA viruses infecting plants and animals.

Keywords Argonaute • Avocado sunblotch viroid • Catalytic RNAs • Central conserved region • Complementary DNA • Delta antigen • Deoxyribonuclease • Dicer-like • Double-stranded RNA • Hepatitis B virus • Hepatitis delta virus • Large isoform delta antigen • MicroRNA • Messenger RNA • Non-protein-coding RNAs • Nuclear-encoded RNA polymerase • Peach latent mosaic viroid • Plastid-encoded RNA polymerase • Polyacrylamide gel electrophoresis • Potato spindle tuber • Potato spindle tuber viroid • RNA polymerase I • RNA polymerase II • RNA polymerase III • Ribonuclease • RNA-induced silencing complex • RNA viruses • Short isoform delta antigen • Small interfering RNA • Tobacco mosaic virus • Transfer RNA • Viroids • Viroid-derived small RN

1 A Historical Overview: Hammerhead Ribozymes in Their Natural Context

To defend themselves against DNA viruses, bacteria have evolved a quite effective strategy based on restriction enzymes, which are DNases that recognize short nucleotide stretches of the virus DNA and proceed to degrade it specifically, while the cell DNA is protected because of previous chemical modifications catalyzed by other enzymes (Arber and Linn 1969). Although bacteria are also invaded by RNA viruses, for reasons that we do not know, they have not evolved restriction RNases, which neither have been described in eukaryotes. Evolution has endowed the latter with alternative defensive barriers: the immunitary system in higher vertebrates, whose final effectors are proteins that specifically bind certain chemical signatures present in the invading virus or cellular pathogen, and the RNA interference (RNAi) system, whose final effectors are small RNAs that in animals and, particularly, in plants also play a defensive role (in addition to other functions) (Fire et al. 1998). A key component of the RNAi system is RISC (*R*NA-*i*nduced *s*ilencing *c*omplex), which somehow acts as a restriction RNase, although with a mechanism quite different from that of restriction DNases from bacteria (Hammond et al. 2000). Intriguingly, small RNAs derived from clustered, regularly interspaced, short palindromic repeats (CRISPR) involved in an adaptable and heritable RNAi-like pathway conferring resistance to phage infection have been recently reported in bacteria (and archaea) [see for a review Gesner et al. (2011)].

Therefore, restriction RNases (using this term sensu lato because such enzymes do not exist sensu stricto) have potential to become a kind of "magic bullet" that could promote the selective degradation of pathogenic RNAs, including those of viruses and viroids. These latter pathogens incite diseases in plants similar to those caused by viruses, but they differ from viruses in structure, function, and evolutionary origin. Viroids are exclusively composed of a small, circular, non-protein-

coding RNA and, consequently, they are "naked genomes" not protected by a capsid protein as in viruses (Diener 2003; Tabler and Tsagris 2004; Flores et al. 2005; Ding 2009; see also Chapter "Viroids: the Smallest Know Infectious Agents Cause Accumulation of Viroid-Specific Small RNAs*"). Moreover, certain viroids properly manipulated can serve as a source of restriction RNases. A brief overview on how viroids replicate will illustrate this point.

Viroid replication occurs through a rolling-circle mechanism (Branch and Robertson 1984). The infecting most abundant viroid RNA, to which the (+) polarity is arbitrarily assigned, hijacks a cellular RNA polymerase for generating oligomeric (−) strands by reiterative transcription of the circular template. These oligomeric (−) RNAs may (1) serve directly as template for producing oligomeric (+) RNAs that are cleaved to unit-length segments by an RNase and circularized by an RNA ligase or (2) be previously cleaved to unit-length segments and ligated to the monomeric (−) forms, which serve as the template for synthesizing oligomeric (+) RNAs, as indicated previously. Therefore, the two pathways of this rolling-circle mechanism, termed asymmetric and symmetric, respectively, demand three RNA catalytic activities: polymerase, nuclease, and ligase, which initially were thought to be of host origin considering that viroids are non-protein-coding RNAs. The polymerase involved in transcription of *Potato spindle tuber viroid* (PSTVd) and other members of the family *Pospiviroidae* that replicate in the nucleus through the asymmetric pathway is RNA polymerase II (Mühlbach and Sänger 1979; Flores and Semancik 1982; Schindler and Mühlbach 1992), while a nuclear-encoded chloroplastic RNA polymerase mediates transcription of *Avocado sunblotch viroid* (ASBVd) and other members of the family *Avsunviroidae* that replicate in the chloroplast through the symmetric pathway (Navarro et al. 2000; Rodio et al. 2007). Intriguingly, although DNA is the physiological template of these nuclear and chloroplastic RNA polymerases, viroids manage to redirect them to transcribe RNA. The information regarding the third enzymatic activity (RNA ligase) involved in viroid replication is limited, but enzymes of this class with nuclear and chloroplastic localization most likely mediate circularization in the families *Pospiviroidae* and *Avsunviroidae*, respectively (Flores et al. 2011).

Going back to the second step of the replication cycle, cleavage of the oligomeric RNAs into their unit-length counterparts is not catalyzed by a host enzyme in the family *Avsunviroidae* but, remarkably, by ribozymes (of the hammerhead class) embedded in both polarity strands. This is a finding with deep functional, evolutionary and applied implications. Of the several ribozyme classes described, hammerhead ribozymes (hereafter abbreviated as hammerheads) initially discovered in viroid and certain satellite RNAs (structurally similar to viroids but functionally dependent on a helper virus) (Prody et al. 1986; Hutchins et al. 1986; Forster and Symons 1987) are the simplest and most studied. Hammerheads are small RNA motifs that at room temperature, physiological pH, and the presence of a divalent cation (generally Mg^{2+}) self-cleave in a specific phosphodiester bond, generating 5′-hydroxyl and 2′,3′ cyclic phosphodiester termini through a transesterification mechanism. The name hammerhead derives from its initial representation in two dimensions recalling the shape of this tool (Fig. 1a), and it is still used despite its tridimensional conformation being different (see below).

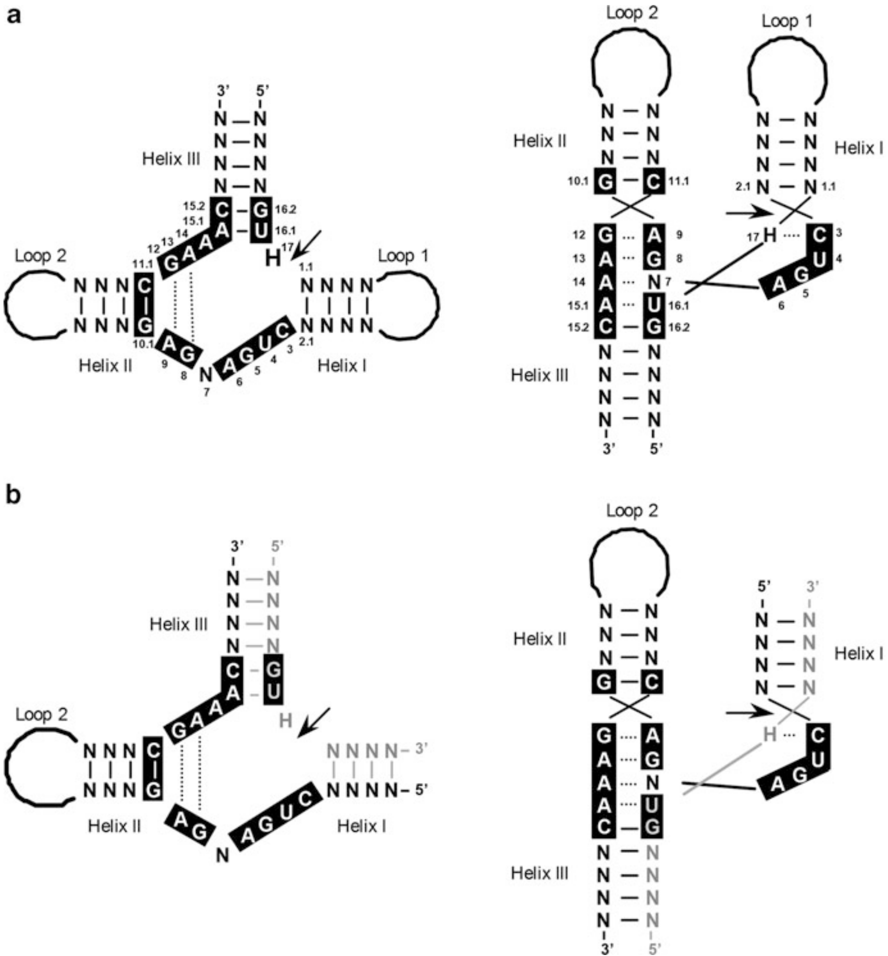

Fig. 1 Structure of the hammerhead ribozyme. (**a**) Schematic representation of the consensus ribozyme and its numbering as originally proposed (*left*) and according to crystallographic data obtained with an artificial hammerhead (*right*). (**b**) Transformation of the natural *cis*-acting format into a *trans*-acting format by disrupting loop 1; the ribozyme and the substrate are denoted with *black* and *gray* fonts, respectively. Nucleotides strictly or highly conserved in most natural hammerheads are on a *black background*, and the self-cleavage site is marked with an *arrow*. N represents any nucleotide, and H represents any nucleotide except G. *Dashes* and *discontinuous lines* indicate Watson–Crick pairs and noncanonical interactions, respectively

2 Manipulating *Cis*-Acting Hammerheads to Act in *Trans*

The consensus hammerhead resulting from a comparative analysis of natural hammerheads present in viroid and satellite RNAs (Flores et al. 2001) (Fig. 1a) reveals that these ribozymes have a strictly conserved central core flanked by three helices (I, II, and III) with minimal sequence restrictions. Site-directed mutagenesis of an artificial hammerhead has shown that changes in the nucleotides forming the

central core are accompanied by severe drops of the catalytic cleavage constant (Ruffner et al. 1990), and crystallographic studies with other artificial hammerheads have unveiled a complex array of interactions between these nucleotides (Pley et al. 1994; Scott et al. 1995). Overall, the 3D structure of the hammerhead resembles a misshapen Y with its basis formed by helix III and the two arms by helices I and II (with the latter being almost colinear with helix III) (Fig. 1a).

It is important to highlight that during viroid replication hammerheads act in *cis* (self-cleaving the RNA in which they are embedded through a single turnover mechanism) (Hutchins et al. 1986). However, a simple experiment with deep implications revealed that a ribozyme of this class can be manipulated to act in *trans* by just splitting it into the ribozyme itself and the substrate (Fig. 1b); to this aim, loop 1, which caps helix I and seemed dispensable, was opened (Uhlenbeck 1987). Two corollaries were inferred from this experiment: (1) it should be possible to design hammerheads targeting specific RNAs for degradation (restriction RNases), the only requisite for which was to identify in the target RNA a GUH [the trinucleotide preceding the self-cleavage site in most natural hammerheads, where H represents any nucleotide except G; see for a review Flores et al. (2001)] and synthesize ribozyme arms complementary to the sequences of the substrate flanking the cleavage site (forming helices I and III) (Fig. 1b) and (2) one molecule of ribozyme could act on several molecules of substrate, through a multiple turnover mechanism, thus increasing the catalytic efficiency. Both predictions were confirmed experimentally (Haseloff and Gerlach 1988), paving the way for a new biotechnological tool ("the genetic scissors"), a kind of antisense RNA with the advantage that a molecule of ribozyme can process more than one molecule of substrate, thus functioning as a true catalyst, in other words, a catalytic antisense RNA.

A suite of additional studies better defined the hammerhead features for efficient and versatile cleavage in *trans*. Illustrative examples include that a helix I of only 3 bp is sufficient for the catalytic activity, showing some advantages of an asymmetric design with helices I and III of different lengths (Tabler et al. 1994); that helix II could be reduced to a few nucleotides, giving rise to what were termed minizymes (McCall et al. 1992); and that there were alternatives to the GUX trinucleotide preceding the cleavage site, like AUA, that were also catalytically active (Kore et al. 1998).

One of the first experiments with this new tool consisted in embedding a hammerhead into an antisense RNA against the RNA of *Human immunodeficiency virus* (HIV): when both RNAs were cotransfected in human cells, the catalytic domain exhibited an inhibitory effect on HIV replication four- to sevenfold higher than a catalytically inactive mutant or the antisense RNA itself (Homann et al. 1993). Similar experiments were performed in plants of *Nicotiana clevelandii* in which an antisense RNA against *Plum pox virus* (PPV), or a variant with an embedded hammerhead, was expressed episomically: following PPV inoculation, plants displayed some delay in the onset of symptoms, which was more pronounced with the catalytic variant (Liu et al. 2000). These results were stimulating but revealed a limited efficiency. Additional studies with antisense RNAs—containing

or not hammerheads against both polarity strands of *Citrus exocortis viroid* (CEVd), a close relative of PSTVd—expressed transgenically in tomato (an experimental host for CEVd), showed a moderate decrease in viroid titer when the plants were challenged-inoculated; moreover, this effect was only detected with the construct against the (−) polarity strand (which accumulates in vivo considerably less than its complementary counterpart), and the catalytic domain did not provide any additional effect (Atkins et al. 1995). In contrast, in a parallel work with PSTVd and potato, a significant resistance was observed in transgenic lines expressing the construct against the viroid (−) strand, with the effect being associated with the catalytic domain because control lines in which this domain was inactivated did not display resistance (Yang et al. 1997). A feasible explanation for these somewhat contradictory results is that PSTVd accumulates to lower levels than CEVd, as also occurs with (−) with respect to (+) strands.

3 A Critical Issue: Colocalization of Ribozyme and Substrate

Although hammerheads offer a promising approach to target and degrade specific RNAs in vivo, important hurdles remain to be overcome for the practical implementation of this technology (Castanotto et al. 2002). To be effective, a hammerhead requires (1) stability against RNases, which for ribozymes that are externally synthesized and then delivered can be enhanced by substituting the 2′-OH of the riboses by other groups; (2) high expression, which for ribozymes synthesized intracellularly is achieved by putting the corresponding constructs under the control of strong promoters; (3) easy access of the ribozyme to the complementary region of its cognate RNA, a question difficult to evaluate considering that this region may adopt stable secondary structures or be associated with proteins; and (4) colocalization of ribozyme and substrate in the same subcellular compartment.

To diminish the effects resulting from the secondary structure of the target RNA around the cleavage site, hammerheads produced intracellularly that are able to unwind double-stranded structures have been designed; to this end, the ribozyme is fused to an RNA motif that interacts and recruits RNA helicases, thus creating a hybrid ribozyme with significantly higher catalytic activity in vivo (Kawasaki and Taira 2002). The issue of colocalizing ribozyme and substrate has been experimentally tackled by coexpressing within the same animal cells two retroviral vectors, one coding for a hammerhead targeting an artificial RNA substrate and the other this same RNA. Due to the common packaging signal, the anticipated outcome was the colocalization of the ribozyme with the substrate, but not with a control RNA not fused to the viral vector. The observed results confirmed this prediction: while the ribozyme reduced more than 90% the titer of the infectious virus fused to the target RNA, no effects were observed with the control RNA (Sullenger and Cech 1993). In a second study using yeast as a model system, a hammerhead was directed to the nucleolus (by fusing it to a small nucleolar RNA), wherein its target RNA

(another nucleolar RNA) was located. The colocalization produced an efficiency close to 100% (Samarsky et al. 1999). Incidentally, this approach could be in principle extrapolated for controlling PSTVd and related viroids that accumulate in the nucleolus.

4 An Unanticipated Participant: Interactions Between Peripheral Loops of Natural Hammerheads Greatly Increase Their Self-Cleavage Activity

Despite extensive research efforts on hammerheads, a key aspect of their mechanism remained unnoticed up to 2003. Until then, most data were obtained with artificial hammerheads operating in *trans* (the format required for ribozymes targeting specific RNAs, which additionally facilitates kinetic analyses in a protein-free medium) and indicated that for their effective functioning in vitro, they needed Mg^{2+} at 5–10 mM, while the concentration in vivo of this cation is about 0.5 mM. As in other similar situations, this paradox pointed to an incomplete understanding of the question. Subsequent results obtained in vitro and in vivo have demonstrated that hammerheads acting in *cis* (the natural format) self-cleave much faster than their *trans*-acting derivatives and that modifications of peripheral loops 1 and 2 of natural hammerheads induce a drastic reduction of their self-cleavage catalytic constant (De la Peña et al. 2003; Khvorova et al. 2003). These data show that, in contrast with the accepted view, regions external to the central conserved core play a key catalytic role in natural hammerheads, suggesting that tertiary interactions between peripheral loops 1 and 2 facilitate sampling of the catalytic-active conformation and permit effective functioning at the low physiological concentrations of Mg^{2+} (Fig. 2a). Other studies support this view and explain why minimal *trans*-cleaving hammerheads require higher concentrations of this cation for adopting the active folding (Rueda et al. 2003; Canny et al. 2004; Penedo et al. 2004). Moreover, the interactions between peripheral loops might be stabilized by proteins, as suggested by the observation that a chloroplastic protein stimulates in vitro, and presumably in vivo, the hammerhead-mediated self-cleavage of a viroid RNA (Daròs and Flores 2002).

Crystallographic analysis of a natural hammerhead present in transcripts of *Schistosoma mansoni* has shown that tertiary interactions between loops far apart from the active site do exist and prime this ribozyme for catalysis at submillimolar Mg^{2+}. More specifically, the new structure confirms previous suggested roles for some conserved nucleotides and offers an explanation for the roles of others that are also conserved (Martick and Scott 2006). Similar analyses extended to the natural hammerhead of satellite RNA of *Tobacco ring spot virus* (sTRSV) (Prody et al. 1986), which is a typical hammerhead with loops 1 and 2 (the former is substituted by an internal loop in the hammerhead from *S. mansoni*), reinforce this view by providing the precatalytic (enzyme–substrate complex) and postcatalytic

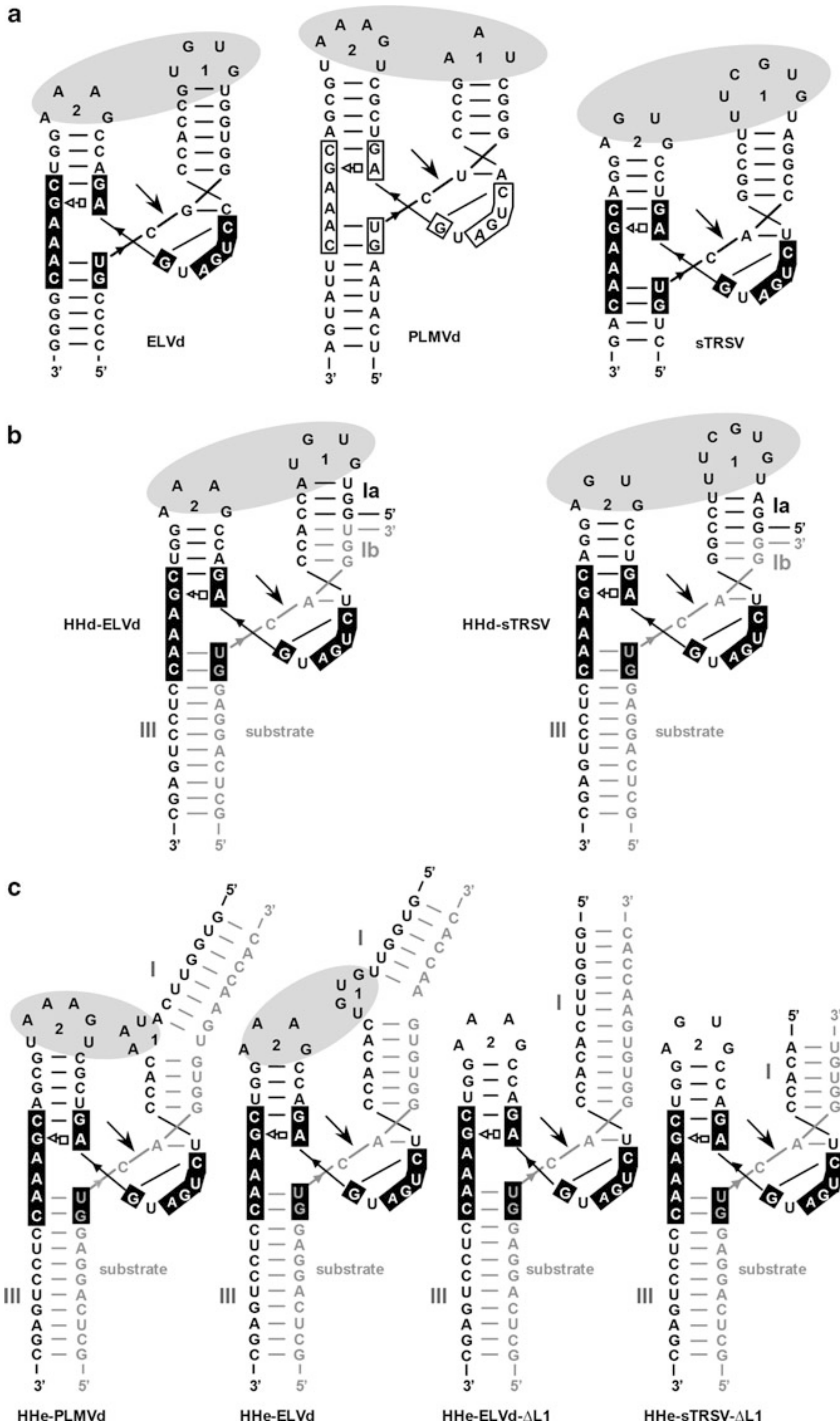

Fig. 2 (continued)

(enzyme–product complex) crystal structures of an active full-length hammerhead RNA that self-cleaves in the crystal (Chi et al. 2008). This same work has also revealed a Hoogsteen pair between an A in stem-loop II and a U in a nonhelical region of stem I that is apparently conserved in most natural hammerheads possibly due to its functional relevance (Chi et al. 2008). Furthermore, examination by NMR spectroscopy of loops 1 and 2 of the hammerheads from *Chrysanthemum chlorotic mottle viroid* (CChMVd) (Navarro and Flores 1997) and dissection of their roles by site-directed mutagenesis, self-cleavage kinetics, and infectivity bioassays have shown that specific nucleotides mediate the interactions between loops 1 and 2; the relevance of these nucleotides is evidenced by their conservation in most natural hammerheads (Dufour et al. 2009).

5 A New Generation of *Trans*-Acting Hammerheads Operating In Vitro and In Vivo at Physiological Concentrations of Magnesium

Besides explaining the low efficiency in vivo of hammerheads with *trans* format, these findings provided momentum for resuming the study of hammerheads under a new perspective. Soon afterward the interactions between peripheral loops became evident, these tertiary stabilizing motifs (TSMs) were incorporated into a new generation of more efficient *trans*-cleaving hammerheads with two distinct designs: (1) extending stem I and including loop 1 as a bulge in the hybridizing arm of this stem (extended format) (Saksmerprome et al. 2004; Weinberg and Rossi 2005) and (2) embedding within stem I the 5′ and 3′ termini of the ribozyme and substrate, respectively (discontinuous format) (Burke and Greathouse 2005) (Fig. 2b, c). Some of these hammerheads are active in vitro at low Mg^{2+} concentration against short RNA substrates. In particular, discontinuous hammerheads (HHd) derived from sTRSV and extended hammerheads (HHe) derived from *Peach latent mosaic viroid* (PLMVd) (Hernández and Flores 1992) are the most efficient when compared with other *trans*-cleaving hammerheads (Saksmerprome et al. 2004; Weinberg and Rossi 2005; Burke and Greathouse 2005).

Fig. 2 (**a**) Self-cleaving hammerheads derived from ELVd, PLMVd, and sTRSV. Motifs conserved in most natural hammerheads are within *boxes*, and self-cleavage sites are marked by *arrows*. *Black* and *white backgrounds* refer to (+) and (−) polarities, respectively. *Dashes* denote Watson–Crick (and wobble) pairs and the *open square–triangle* a Hoogsteen/sugar edge interaction. *Ovals* represent the proposed tertiary interactions between loops 1 and 2. (**b**) Schematic representation of the complex formed by discontinuous hammerheads (HHd) derived from ELVd and sTRSV and their short RNA substrates. Hammerhead and substrate nucleotides are shown with *black* and *gray* fonts, respectively. Ia and Ib refer to the distal and proximal halves of stem I, respectively. (**c**) Schematic representation of the complex formed by extended hammerheads (HHe) derived from PLMVd, ELVd, and sTRSV and their short RNA substrates. HHe-PLMVd and HHe-ELVd hammerheads contain TSMs, while the minimal HHe-ELVd-ΔL1 and HHe-sTRSV-ΔL1 hammerheads lack these motifs. Adapted from Carbonell et al. (2011)

However, other natural hammerheads like those of *Eggplant latent viroid* (ELVd) (Fadda et al. 2003) have not been adapted to a *trans* design, despite displaying higher self-cleavage rates than other natural hammerheads at very low Mg^{2+} concentrations (Carbonell et al. 2006) and despite appearing particularly convenient for the discontinuous format (because their long stem I of seven base pairs should facilitate substrate binding and folding of loop 1) (Fig. 2a).

In the remaining part of this chapter, we will focus on our recent efforts aimed at designing discontinuous and extended hammerheads derived from ELVd, PLMVd, and sTRSV with TSMs (Fig. 2b, c) (Carbonell et al. 2011). We have first examined the ability of these hammerheads to catalyze the *trans*-cleavage in vitro of short RNA substrates and of a long and highly structured RNA containing the complete sequence of the pathogen PSTVd (Diener 1971; Gross et al. 1978). Then we have selected an extended PLMVd-derived hammerhead with natural TSMs, which displays in vitro the highest cleavage rate, for transient expression bioassays in *Nicotiana benthamiana* plants to examine its activity in vivo against PSTVd infection.

6 *Trans*-Cleavage In Vitro of Short RNA Substrates by Discontinuous and Extended Hammerheads

Previous studies have shown that *cis*-acting ELVd hammerheads with a GUC (or AUC) trinucleotide preceding the self-cleavage site are the most active at very low Mg^{2+} concentration (Carbonell et al. 2006). Therefore, we took these hammerheads as a starting platform for designing HHd with preserved TSMs, for *trans*-cleaving a short fragment of the PSTVd minus (−) strand with a GUC target site. Analyses of several HHd revealed that the most efficient at low Mg^{2+} were derived from natural hammerheads in which the sequences of loops 1 and 2 remain unaltered. Unexpectedly, another ELVd-derived ribozyme with an artificial loop 1 (AAAA) was also active, suggesting that alternative tertiary interactions between artificial loops 1 and the wild-type loop 2 might promote cleavage at submillimolar Mg^{2+}. PLMVd-derived hammerheads were not tested because they are inappropriate for the discontinuous format (their stem I has only five base pairs) (Fig. 2a).

Because experiments with short RNA substrates have reported that extended hammerheads derived from PLMVd are particularly efficient (Saksmerprome et al. 2004; Weinberg and Rossi 2005), we designed a variant (HHe-PLMVd) targeting a short RNA fragment of the PSTVd (−) strand. This HHe-PLMVd, with natural loops 1 and 2 to preserve their interactions, displayed a high cleavage constant even at 0.1 mM Mg^{2+}. We next examined two extended hammerheads, HHe-ELVd (also with loops 1 and 2 as in the natural ribozyme) and HHe-ELVd-ΔL1 (a minimal hammerhead in which loop 1 was deleted); the higher catalytic constant of the former highlighted the need of TSMs for activity at submillimolar Mg^{2+}. An additional control with HHe-sTRSV-ΔL1, a modified version of the sTRSV ribozyme without TSMs but with some activity in vivo when stably expressed in transgenic potato plants (Yang et al. 1997), confirmed this view.

Altogether these experiments showed that some ELVd-derived hammerheads in discontinuous format target for cleavage short RNA substrates. However, their lower efficiency when compared with a sTRSV-derived hammerhead suggests that a hybridizing stem of only three base pairs ensures substrate binding. On the other hand, extended versions of the ELVd hammerhead were active against a short RNA substrate, but their catalytic constant, lower than that derived from the PLMVd hammerhead, indicated that the simple transposition of loop 1 to adapt a hammerhead to the *trans* format may not preserve a high catalytic activity (Weinberg and Rossi 2005).

7 *Trans*-Cleavage In Vitro of a Highly Structured RNA by Discontinuous and Extended Hammerheads

Early attempts to target long RNA substrates with minimal hammerheads met with limited success: the in vitro *trans*-cleavage constants were 100-fold lower than those obtained for short RNA substrates, a likely consequence of catalytically inactive complexes being formed between the substrate and the ribozyme (Hormes and Sczakiel 2002), or of folded substrates restricting access of the ribozyme to the vicinity of the cleavage site (Campbell et al. 1997). As indicated above, discovery of TSMs in natural hammerheads (De la Peña et al. 2003; Khvorova et al. 2003) prompted their incorporation into ribozymes with discontinuous and extended formats for enhanced activity at low Mg^{2+} concentration. However, these studies have been performed in vitro and against short RNA substrates, with only one extended PLMVd-derived hammerhead having been tested against a long RNA (a 258-nt fragment of the HIV genome) (Saksmerprome et al. 2004).

To provide additional data in this context, the hammerheads described in the previous section were next examined in vitro against a full-length PSTVd (−) RNA (359 nt) with a GUC target site and two vector tails of 17 and 88 nt; this RNA adopts a rodlike conformation according to previous studies and our own RNase T1 probing experiments. In contrast with data obtained for the short RNA substrates, most of the HHd derived from ELVd catalyzed cleavage of the long substrate more efficiently than their sTRSV-derived counterpart. Moreover, the higher cleavage rate of a variant in which the U–A base pair closing loop 1 was substituted by a stronger C–G base pair supports that the stability of stem Ib is critical for preserving the TSMs. Also worth noting is that two of the ELVd-derived hammerheads with artificial loops 1 (UACG and AAAA) were active at submillimolar Mg^{2+}, thus suggesting that alternative TSMs between artificial sequences of loop 1 and the natural loop 2 might promote cleavage as reported for a sTRSV-derived hammerhead with an artificial tetraloop (Burke and Greathouse 2005). Because the sequences of natural hammerheads have been selected as a compromise between self-cleavage and other functions that they additionally mediate (De la Peña and

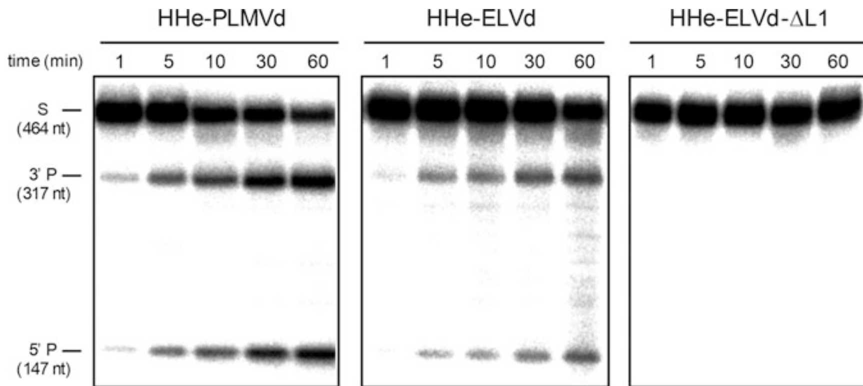

Fig. 3 Analysis by denaturing PAGE (5%) and autoradiography of *trans*-cleavage in vitro (at 0.1 mM Mg^{2+}) of PSTVd (−) RNA catalyzed by three extended hammerheads (HHe) derived from PLMVd and ELVd (see Fig. 2c). The positions and size of the substrate (S) and of the resulting 3′ and 5′ cleavage products (P) are indicated. HHe-ELVd-ΔL1 refers to the minimal hammerhead without TSMs. Adapted from Carbonell et al. (2011)

Flores 2001; Carbonell et al. 2006), it is not surprising that artificial sequences forming part of the TSMs (Khvorova et al. 2003; Saksmerprome et al. 2004) or the catalytic core (Carbonell et al. 2006) could even enhance cleavage in a *trans* format.

The most efficient extended hammerhead against the long RNA substrate, especially at submillimolar Mg^{2+}, was derived from PLMVd (Fig. 3), in line with previous in vitro selection studies at low Mg^{2+} concentration in which a PLMVd-derived hammerhead with only two transitions in loop 2 with respect to the natural variant was selected for the fastest self-cleavage (Saksmerprome et al. 2004). The nucleotides forming the asymmetric bulging loop of the HHe-PLMVd most likely generate TSMs resembling those existing in the natural hammerhead, because a smaller bulging loop (of three nucleotides) permits less alternative interactions than in the HHe-ELVd (with a bulging loop of four nucleotides). Supporting this view, extended hammerheads derived from sTRSV and CChMVd, with bulging loops of seven nucleotides, also display low catalytic efficiency (Weinberg and Rossi 2005).

8 *Trans*-Cleavage In Vivo of a Viroid RNA by an Extended PLMVd-Derived Hammerhead

Considering that at low Mg^{2+} concentration the HHe-PLMVd displayed the best activity against short and long RNAs in vitro, this hammerhead was chosen for further evaluation *in planta*. For this purpose, two cultures of *Agrobacterium tumefaciens* transformed with constructs expressing the ribozyme and the

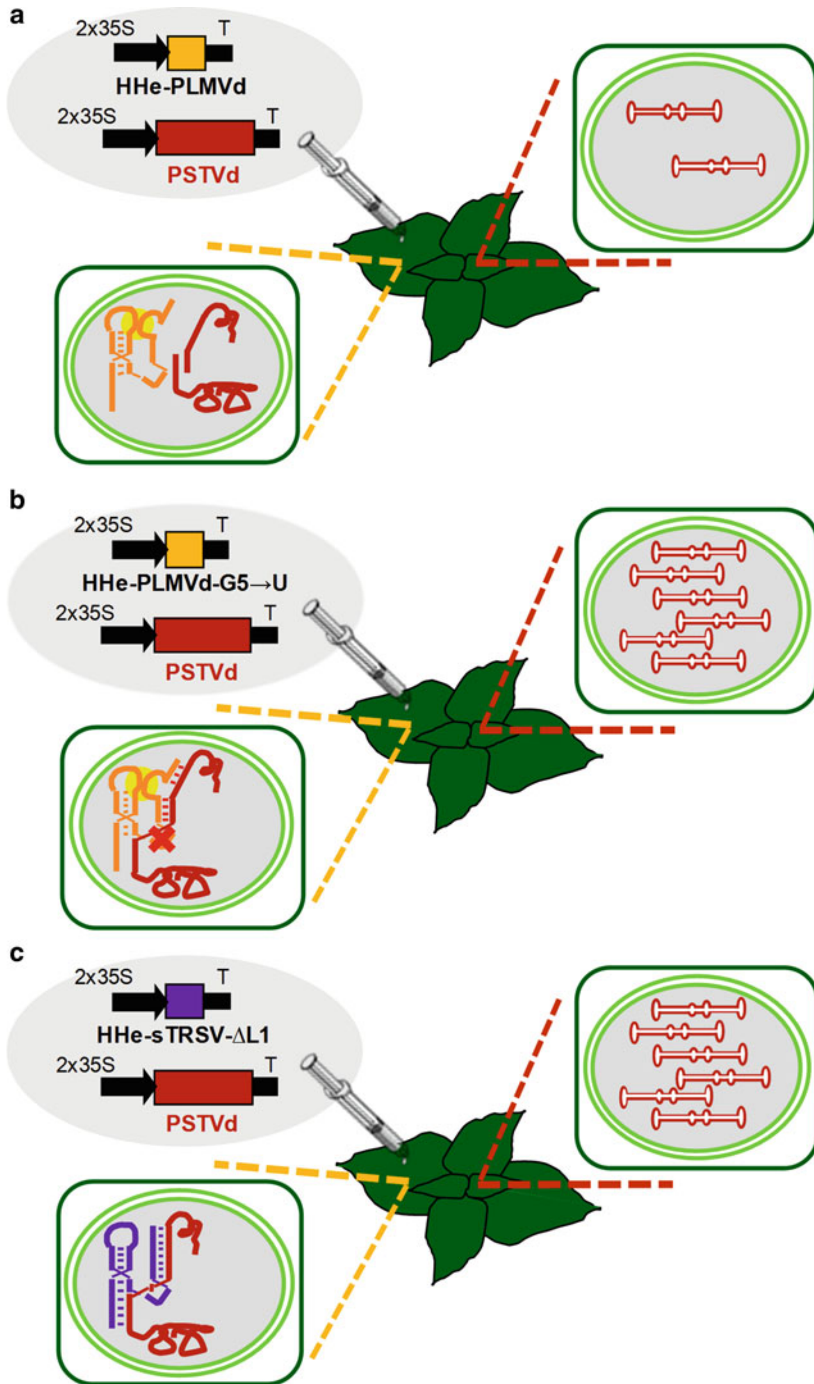

Fig. 4 Schematic representation of the mode of action of three extended hammerheads targeted against the PSTVd (−) RNA: the catalytically active HHe-PLMVd (**a**) and the inactive variants

substrate were coinfiltrated in *N. benthamiana* leaves (Fig. 4). Regarding the substrate, two constructs were used: mPSTVd(−), which results in a noninfectious monomeric PSTVd (−) RNA, and dPSTVd (−), which generates a head-to-tail dimeric PSTVd (−) RNA that triggers replication through the asymmetric variant of the rolling-circle mechanism (see above). Regarding the ribozyme, besides the catalytically active HHe-PLMVd, two other inactive variants were assayed: HHe-PLMVd-G5 → U, in which the CUGA box of the central conserved core was mutated into CUUA (resulting in a catalytically inactive hammerhead), and HHe-sTRSV-ΔL1, in which loop 1 was deleted (disrupting the TSM).

Bioassays coexpressing each of the three hammerheads with the noninfectious mPSTVd(−) RNA substrate revealed that only the HHe-PLMVd was active in vivo, strongly suggesting that the lower accumulation of the PSTVd transcript most likely results from ribozyme-mediated cleavage and that TSMs are critical in this respect (Fig. 4). Moreover, this same hammerhead interfered with viroid infection when coexpressed with the infectious dPSTVd (−) RNA, indicating that it may target the primary dimeric transcript and perhaps also the oligomeric (−) replicative intermediates (Fig. 4). Because previous results with a minimal hammerhead similar to HHe-sTRSV-ΔL1 only conferred resistance against PSTVd in some potato transgenic lines, but not in transgenic tomato (Yang et al. 1997), the constitutive expression in transgenic plants of an improved ribozyme like HHe-PLMVd may control PSTVd more efficiently.

9 Concluding Remarks and Outlooks

The use of bioinformatic tools for searching databases has recently led to another unexpected turn: the presence of hammerheads in certain transcripts from newts, crickets, and schistosomes is not the exception, as initially considered, but a rather common situation (De la Peña and Garcia-Robles 2010). Hammerheads are widely spread throughout the biological scale, with their genesis most likely involving retrotransposition events and their location in host genomes strongly supporting a regulatory role. Although a careful dissection of these novel ribozymes is still pending, they provide an arsenal of new variants for proper conversion into the *trans*-acting format.

Fig. 4 (continued) HHe-PLMVd-G5 → U (with a mutation in the catalytic core) (**b**) and HHe-sTRSV-ΔL1 (with the TSM disrupted) (**c**). Within each panel: (1) the *upper left oval outlines* the two constructs expressing the ribozyme and the substrate from two *A. tumefaciens* cultures coinfiltrated in *N. benthamiana* leaves (2×35S and T refer to the promoter and terminator, respectively); (2) the *lower left rectangle* details the ribozyme (in *orange* or *blue*, with the TSM highlighted with a *yellow background*) and the substrate (in *red*), and whether or not the latter is cleaved; and (3) the *right rectangle* summarizes the observed effect on viroid titer. The ribozyme and the genomic viroid RNA (represented by a rodlike structure) are not at the same scale

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