Plant Biotechnology Journal (2003) **1**, pp. 423–435 Doi: 10.1046/j.1467-7652.2003.00039.x

Development of an efficient *cis-trans-cis* ribozyme cassette to inactivate plant genes

Frédéric Bussière[1,2], Sylvain Ledû[2], Marianne Girard[1], Maryse Héroux[1], Jean-Pierre Perreault[2,*] and Daniel P. Matton[1,†]

[1]*Institut de Recherche en Biologie Végétale, Département de Sciences Biologiques, Université de Montréal, 4101 rue Sherbrooke Est, Montréal, QC, H1X 2B2, Canada;* [2]*Groupe ARN/RNA Group, Département de Biochimie, Université de Sherbrooke, Sherbrooke, QC, J1H 5N4, Canada*

Received 18 June 2003;
revised 2 July 2003;
accepted 4 July 2003
Correspondence *(fax: +1 819 564 5340; e-mail Pierre.Perreault@USherbrooke.ca), †(fax: +1 514 872 9406; e-mail dp.matton@umontreal.ca)

Keywords: catalytic RNA, hammerhead ribozyme, plant endogenous gene, self-cleaving cassette.

Summary

Inactivation of a targeted gene is one of the main strategies used to understand their precise cellular role. In plants, apart from chemical or physical mutagenesis and random insertions of DNA elements followed by screening for a desired phenotype, the most common strategy to inhibit the expression of a given gene involves RNA silencing. This can be achieved either through antisense suppression, sense over-expression leading to co-suppression, or expression of double-stranded DNA constructs (dsRNA). The use of ribozymes to inhibit gene product accumulation has only been occasionally attempted, mainly because of the more complex genetic engineering procedure involved, although the specificity of ribozymes can be an important factor when targeting close members of a gene family. We report here the development of a new *cis*-acting ribozyme cassette for the production of RNAs with desired termini. Attention to many details has been brought in order to provide a powerful procedure for plant application. For example, ultrastable GNRA tetraloops were substituted for both loops II and III of *cis*-acting hammerhead sequences, thereby favouring folding into the catalytically active structure that results in the self-cleavage of all transcripts. We demonstrate the usefulness of this cassette by producing a ribozyme that cleaves *in trans*, originally embedded in the *cis*-acting self-cleaving cassette. The activity of the *cis-trans-cis* construct, was demonstrated both *in vitro* and *in vivo*, in transgenic plants with the specific cleavage of an mRNA encoding a 2-oxo-glutarate-dependant dioxygenase predominantly expressed in pistils tissues and in leaves, from the wild potato *Solanum chacoense*.

Introduction

The functional analysis of plant genes has greatly benefited from the availability of mutant lines, mainly in model species, and from the ability to down-regulate the expression of specific genes through antisense suppression, sense over-expression leading to co-suppression, or expression of double-stranded RNA constructs (dsRNA) leading to RNA-induced gene silencing (Fagard and Vaucheret, 2000; Mlotshwa *et al*., 2002; Tijsterman *et al*., 2002; Vaucheret *et al*., 2001; Waterhouse and Helliwell, 2003). Recently, immunomodulation (i.e. the use of *in vivo* expressed antibodies), has also been shown to inhibit a protein's activity through specific antibody-antigen recognition and binding (Conrad and Manteuffel, 2001; De Jaeger *et al*., 2000; Jobling *et al*., 2003). Another attractive strategy for modulating RNA abundance is the use of catalytic RNA molecules (ribozymes) that specifically cleave target RNAs. Although less frequently used in plants, mainly because of design complexity, catalytic RNA molecules have many advantages (Vaish *et al*., 1998). Firstly, because the recognition stem (stems I and III in the case of hammerhead ribozyme) can be very short, this considerably increases the specificity towards the target RNA and also increases the turnover rate by decreasing the energy necessary to dissociate the ribozyme after cleavage (Lewin and Hauswirth, 2001; Michienzi and Rossi, 2001). This also enables

ribozymes to distinguish between highly similar genes in multigene families. However, this is not the only feature that contributes to defining the substrate specificity – the structure of both the ribozyme and the targeted mRNA are important. Secondly, ribozymes are true enzymes, thus one molecule can successively cleave several substrate RNA molecules. Thirdly, folding of the ribozyme into a specific motif (e.g. hammerhead, hairpin, *delta* ribozyme) increase its stability and thus its processivity. In plants, only a few attempts have been made to use the potential of hammerhead ribozymes (originating from the *cis*-acting RNA catalytic motif essential for the rolling circle replication of some plant RNA species, i.e. viroids and RNA satellite of plant virus (Symons, 1997)), to target endogenous mRNA *in planta* (Borovkov *et al*., 1996; McIntyre *et al*., 1996; Merlo *et al*., 1998). Moreover, studies involving the use of protoplasts have been performed to show the *in vivo* activity of ribozymes against co-expressed markers or reporter constructs (Perriman *et al*., 1993, 1995; Steinecke *et al*., 1992, 1994). Most ribozymes expressed *in planta* targeted plant viruses as a means of conferring virus resistance (Atkins *et al*., 1995; de Feyter *et al*., 1996; Han *et al*., 2000; Huttner *et al*., 2001; Kwon *et al*., 1997; Liu *et al*., 2000; Yang *et al*., 1997). Although the use of the ribozyme is clearly effective in cleaving RNA targets *in vivo*, many parameters render their use difficult. Their design needs to be optimized and proof of activity *in vitro* cannot predict if the ribozyme will effectively be stable *in vivo*. RNA target accessibility is also different *in vivo* due to the presence of RNA binding proteins. Furthermore, the insertion of ribozymes in longer transcripts introduces extra sequences that often result in RNAs with limited biological activity, or one showing structural heterogeneity. For example, the activity of *trans*-acting hammerhead ribozymes can be impaired by additional sequences at the 5′ and 3′ termini of the catalytic core sequence (Bertrand *et al*., 1994; Denman, 1993; Fedor and Uhlenbeck, 1990; Ruiz *et al*., 1997). The additional sequences either reduce the ability of the ribozyme to bind to a given target (by allowing non-specific hybridization of the ribozyme) or result in non-productive folding. *Cis*-acting ribozyme cassettes have often been used in animal systems to produce RNA molecules with the appropriate termini (Altschuler *et al*., 1992; Feng *et al*., 2001; MacKay *et al*., 1999; Ruiz *et al*., 1997). In this procedure a ribozyme gene is sandwiched between two self-cleaving RNA motifs (i.e. *cis*-acting catalytic RNA). During transcription the *cis*-acting catalytic RNA self-cleave, releasing a ribozyme which can subsequently acts *in trans*. Unfortunately, in most cases, self-cleavage efficiency only ranges from 50% to 80% (Feng *et al*., 2001; MacKay *et al*., 1999). Consequently, an important percentage of the RNA molecules still contain extra sequences yielding inactive ribozymes.

Our objective in this study was to overcome this limitation by developing a new *cis*-ribozyme cassette which self-cleaved all transcripts. Ultrastable GNRA tetraloops, which are known for their contribution to the folding of various RNA molecules (Varani, 1995), were included as loops II and III of both *cis*-acting hammerhead sequences in order to favour the adoption of the catalytically active structure (see Figure 1A). Selection of the transformants expressing the ribozyme cassette was also made easy by embedding the ribozyme cassette into the 3′ untranslated region of a marker gene. As an illustration of the potential of our *cis-trans-cis* cassette, we describe the production of a ribozyme that exhibits cleavage activity *in vitro*, as well as in transgenic plants, *in trans*, of an mRNA encoding a developmentally regulated dioxygenase expressed in pistils and in leaves of the wild potato *Solanum chacoense* Bitt (Lantin *et al*., 1999).

Experimental procedures

Materials

Restriction enzymes, DNase I (RNase-free), T4 DNA ligase, RNA Guard (RNase free), *Escherichia coli* RNase H, calf intestine alkaline phosphatase, T4 polynucleotide kinase, T7 sequencing kit, Sephadex G-50 gel matrix (DNA grade), T7 RNA polymerase and T3 RNA polymerase were purchased from Amersham Biosciences. Radiolabelled nucleotides [α-^{32}P]GTP (3000 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from New England Nuclear, and [α-^{32}P]dCTP (3000 Ci/mmol) from ICN Biochemicals.

Ribozyme and catalytic RNA vector construction

The SPP2 dioxygenase was originally isolated by subtractive hybridization to characterize the genes involved in pollen–pistil interactions and early fertilization events (Lantin *et al*., 1999). Subtracted cDNA libraries were made using mRNA from pollinated pistils 48 h post-pollination, from which were subtracted mRNAs common to mature unpollinated pistils. The pool of subtracted cDNAs was then PCR amplified and used to screen 48 h and 96 h post-pollination *Solanum chacoense* pistil cDNA libraries made in the λZap-pBK vector (Stratagene). The SPP2 dioxygenase cDNA was subsequently subcloned into pBluescript II KS (+/–) in order to place the dioxygenase cDNA downstream of the T7 RNA polymerase promoter for further *in vitro* run-off transcription.

Both the pRz1 and pRz1-G5U plasmids carry ribozyme sequences in pBluescript II KS (+/–) (Stratagene). The

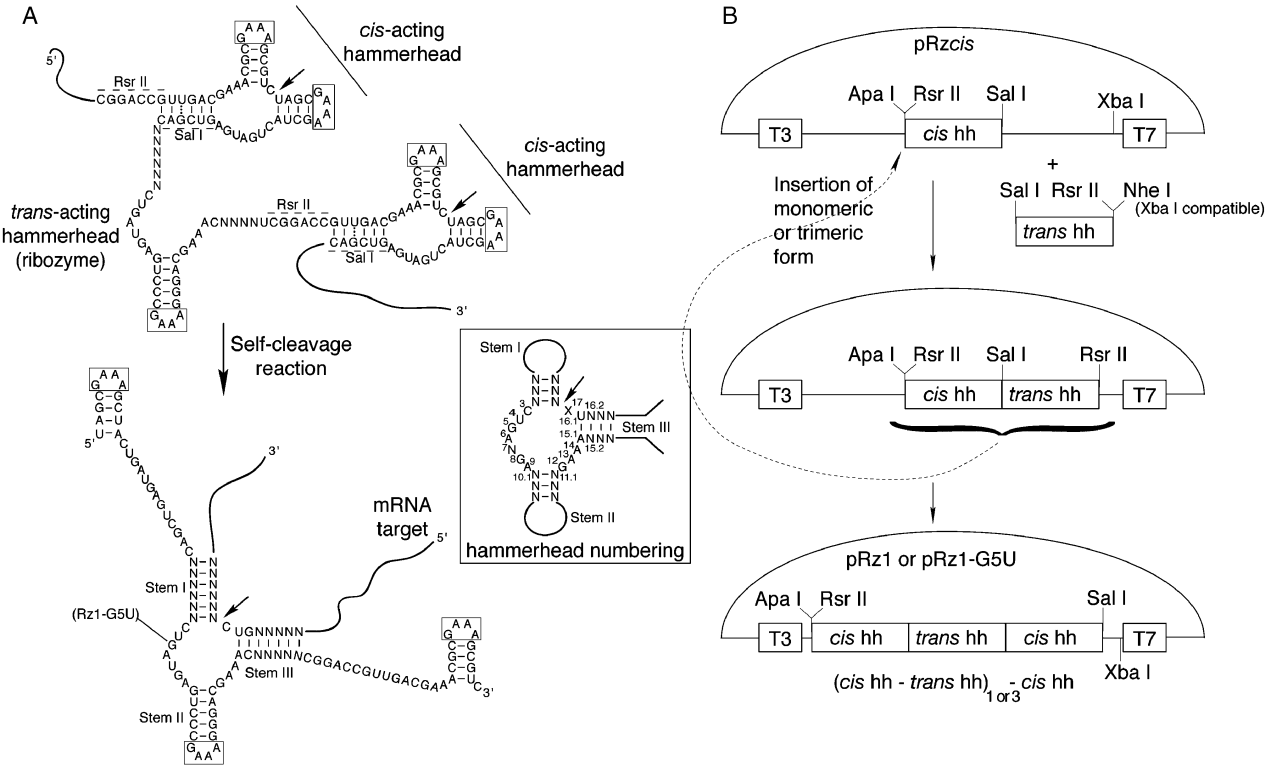

Figure 1 (A) Schematic representation of *cis*- and *trans*-cleavage. The transcription of the *cis-trans-cis* cassette produces an RNA that has to self-cleave at two sites in order to release the *trans*-acting hammerhead motif. Subsequently, the *trans*-acting motif hybridizes with, and cleaves the targeted mRNA. Arrows indicate the cleavage sites. Boxes show the ultrastable GNRA tetraloops. The inactive mutant used as a control is represented by Rz-G5U. Non-Watson-Crick GU wobble base pairs are represented by small dotted lines (⋯). (B) Schematic of the cloning procedure to create the *cis-trans-cis* catalytic RNA cassette. The classical hammerhead motif numbering is shown in the small inset.

ribozymes were cloned in pBluescript II KS (+/–) under the control of the T3 RNA polymerase promoter. Briefly, the *cis*-acting ribozyme was synthesized using two oligonucleotides corresponding to the hammerhead sequences and harbouring the *Apa*I, *Rsr*II and *Sal*I restriction sites: 5′-GGGGGCCC-GGACCGTTGACGAAACGCGAAAGCGTCTAGCGAAAGCT-ACTGATGAGTCGACGCG-3′ and its reverse complement. After annealing of the oligonucleotides, the complex was gel purified, digested with the *Apa*I and *Sal*I restriction enzymes, and cloned into a linearized pBluescript vector. The resulting plasmid pRz*cis* (Figure 1B) was digested at the *Sal*I and *Xba*I sites, and a duplex, generated by annealing oligonucleotides corresponding to a *trans*-acting hammerhead with sequences complementary to the SPP2 dioxygenase (5′-GCGGTCGA-CGTGCTTCTGATGAGTCCGAAAGGACGAAACCAGTTCG-GACCGGCTAGCCG-3′ and its reverse complement), was ligated into the vector yielding a *cis-trans* cassette (Figure 1B). The latter construct was digested with *Rsr*II and cloned into pRz*cis* to yield the *cis-trans-cis* construct in which the *trans*-acting ribozyme is surrounded by two *cis*-acting hammerhead sequences (Figure 1B). This construct (pRz1) is used for *in vitro* self-cleaving assays and to produce the *trans*-acting ribozyme for kinetic experiments. The monomer coming from the *Rsr*II digestion of pRz*cis* was also self-ligated to produce a multimeric form. The fact that *Rsr*II is not a palindromic sequence results in directional intermolecular ligations. The trimeric form was gel-purified and cloned into pRz*cis*, leading to a construct that can generate three *trans*-acting ribozymes per transcript upon self-cleavage of the *cis*-acting hammerheads (Figure 1). The trimer was PCR amplified with primers having tails containing a *Bgl*II (forward) or a *Pst*I + *Sac*I (reverse) sites [5′-GAGAAGATCTGCAGGGAACAAAA-GCTGGG (forward), 5′-AGGCTGCAGAGCTCAAGCTTAT-CGATACCG-3′ (reverse)]. The product was subcloned into pBluescript II KS (+/–) at the *Pst*I and *BamH*I (compatible with *Bgl*II) sites so that *Sac*I restriction sites are found on each side of the insert. The same strategy was followed for the construction of the active ribozyme and for the G5U inactive form (pRz1-G5U is the mutated form corresponding to the *cis-trans-cis* cassette pRz1).

For the *in vivo* assay, the trimeric cassettes were excised with *Sac*I and cloned at the *Sac*I site into the pBin35GUS transformation vector (Jefferson *et al.*, 1987). This vector contains the β-glucuronidase (GUS) reporter gene followed

by the nopaline synthase (NOS) terminator, and transcription was driven from the CaMV 35S promoter with doubled enhancers. The trimeric cassettes were inserted between the GUS gene and the NOS terminator to produce the plasmids pBin35GUS/Rz1 and pBin35GUS/Rz1-G5U. Each step of the cloning protocol was verified by complete sequencing of the inserts, and the transformation vectors contained tandemly arranged trimers of the *cis-trans-cis* ribozyme in the same transcriptional orientation in order to increase the number of ribozyme copies produced per GUS transcript. For the production of sense over-expressing lines and antisense lines, the complete SPP2 dioxygenase cDNA (Lantin *et al*., 1999) was inserted in the appropriate orientation in the same vector but without the GUS marker gene.

RNA synthesis

Dioxygenase mRNA was produced by transcription from 3 μg of a *Xho*I-linearized pDXG, while ribozymes were produced by transcription from 5 μg of a *Bst*XI-linearized pRz1 and pRz1-G5U. *In vitro* transcriptions were performed using either T3 or T7 RNA polymerase with or without 50 μCi [α-^{32}P]GTP under conditions described previously (Ananvoranich and Perreault, 1998). The mRNA and ribozyme transcripts were purified by electrophoresis through 5 and 10% denaturing polyacrylamide gels (PAGE) 19 : 1, acrylamide to bisacrylamide, respectively, using 50 mM Tris-borate pH 8.3/1 mM EDTA/7 M Urea solution as the running buffer. The reaction products were visualized either by autoradiography, or by ultraviolet shadowing over a fluorescent thin-layer chromatography plate. The bands corresponding to the correct sizes were cut out, and the transcripts eluted from these gel slices by incubating overnight at 4 °C in 0.5 M ammonium acetate, 0.1% SDS solution. The eluted transcripts were then ethanol precipitated, ethanol washed, dried and the quantity determined by spectrophotometry at 260 nm or ^{32}P counting after dissolving in ultrapure water. Small substrates were *in vitro* transcribed from templates (2 μg) formed by two annealed oligonucleotides, and were then purified on 20% denaturing PAGE gels as described above.

5′-end labelling of RNA

Purified small substrates (10 pmol) were dephosphorylated in 20 μL reaction mixtures containing 200 mM Tris-HCl, pH 8.0, 10 units of RNA guard, and 0.2 units of calf intestine alkaline phosphatase at 37 °C for 30 min, and were purified by extracting twice with an equal volume of phenol : chloroform (1 : 1), and ethanol precipitation. Dephosphorylated transcripts (1 pmol) were end-labelled in a mixture containing 1.6 pmol [γ-^{32}P]ATP, 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM KCl and three units of T4 polynucleotide kinase at 37 °C for 45 min. The small substrates were then purified on 20% denaturing PAGE gel as described above. The concentrations of the labelled transcripts were adjusted to 0.01 pmol/μL by the addition of water.

Oligonucleotide probing

DNA oligonucleotides complementary to the potential target sites were purchased from Gibco-BRL. Randomly labelled SPP2 mRNA (≈ 10 000 c.p.m., ≈ 3 nM) and unlabelled oligonucleotides (250 μM) were hybridized together for 10 min at 25 °C in a solution containing 50 mM Tris-HCl pH 7.5 and 10 mM $MgCl_2$ in a final volume of 14 μL. *Escherichia coli* ribonuclease H (RNase H, 0.2 units/μL) was then added and the reaction incubated at 37 °C for 20 min. The reaction was stopped by the addition of a stop solution (3 μL of 97% formamide, 10 mM EDTA, 0.025% bromophenol blue and 0.025% xylene cyanol), fractionated on 5% denaturing PAGE gels, visualized by autoradiography and finally quantified using a PhosphoImager (Molecular Dynamics).

Cleavage reactions

Unless otherwise stated, cleavage reactions were carried out in 20 μL mixtures containing 50 mM Tris-HCl (pH 7.5) and 50 mM $MgCl_2$ at 37 °C as described previously (Ananvoranich and Perreault, 1998). Prior to the reaction, trace amounts of ^{32}P-5′-end-labelled substrate and non-radioactive ribozyme (200 nM) were mixed together and denatured at 95 °C for 2 min, chilled on ice for 2 min, and finally equilibrated at 37 °C for 5 min. The reaction was then initiated by addition of the buffer. Aliquots (2 μL) were removed at various time points and quenched by the addition of 8 μL ice-cold formamide/dye mixture (95% formamide, 10 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). Substrate and products were separated on a 20% PAGE gel and analysed with an InstantImager (Packard). The extent of the cleavage reaction was measured from the amount of radioactivity left in the substrate bands compared with that of the 5′ product bands. At least two independent experiments were performed for each time-course. The fractions of substrate cleaved were determined and the rates of cleavage (k_{obs}) were obtained from fitting the data to the equation $A_t = A_8 (1 - e^{-kt})$ where A_t is the percentage of cleavage at time t, A_8 is the maximum cleavage (i.e. cleavage end-point) and k is the rate constant (k_{obs}).

Plant transformation and *in vivo* assay

Both pBin35GUSRz1 and pBin35GUSRz1-G5U were transferred into wild potato *Solanum chacoense* Bitt plants using *Agrobacterium tumefaciens* as described previously (Horsch *et al*., 1985; Matton *et al*., 1997). Transgenic plants were initially selected on a media containing the antibiotic kanamycin. To select plants that were expressing the ribozyme constructs, a single leaflet from *in vitro* grown transgenic plants was ground in a 1.5 mL test tube and a simple non-quantitative GUS colorimetric assay was performed (Gallagher, 1992). Plants expressing variable amounts of the GUS transgene were thus selected without normalization or exact quantification of the GUS activity. As the ribozyme is located just after the GUS ORF in the constructs, this ensures that a significant amount of the ribozyme was expressed. After 12 weeks, total RNA was extracted from the leaves of several plants as described previously (Jones *et al*., 1985). The RNA concentrations were determined by measuring their absorbance at 260 nm, and verified (and adjusted, if necessary) by agarose gel electrophoresis and ethidium bromide staining. Equal loading of total RNA on RNA gel blots was confirmed using a PCR amplified 1 kb fragment of the *Solanum chacoense* 18S RNA as probe. Probes for both the 18S rRNA and dioxygenase mRNA were synthesized from random-labelled isolated DNA inserts using α-^{32}P-dCTP as labelled nucleotides, and the RNA gel blot hybridizations performed as described previously (Lantin *et al*., 1999).

Results

Hammerhead design and cloning

The hammerhead is a catalytic RNA motif (Figure 1, inset) that promotes self-cleavage at a specific site (Figure 1, arrow). It is made of three stem-loops and three conserved domains. Variation in the identity of some nucleotides in the latter can have critical effects on the catalytic properties of the hammerhead (Vaish *et al*., 1998). Among them are the critical G10.1–C11.1 base pair (Clouet-d'Orval & Uhlenbeck, 1997; Tuschl and Eckstein, 1993) and bases found at position 7, 16.2 and 17. We took advantage of current knowledge on the catalytic properties of the hammerhead motif for the design of *cis*- and *trans*-acting hammerhead sequences (see below).

For *cis*-acting hammerhead ribozymes, the length of stems I and II was chosen to avoid the inhibition of catalytic activity that occurs *in vitro* when both stems I and II are longer than their critical size, which is 5 bp and 4 bp, respectively (Clouet-d'Orval & Uhlenbeck, 1997; Tuschl and Eckstein, 1993). In one case, the stem I was composed of only 3 bp, but this is not a general rule (Tabler *et al*., 1994). GNRA tetraloops were also added for their known high intrinsic stability, favouring the folding of the catalytically active structure (unpublished data, D. Lévesque, K. Fiola, and J.-P. Perreault). Finally U7 as well as the G16.2 and C17 found in the NUX triplet allow high k_{obs} values (Fedor and Uhlenbeck, 1992). Sequences in stem II also include restrictions sites for the cloning procedure (see below). The design was designed to obtain highly active hammerhead motifs, as well as to limit the length of the spacer between the target recognition sequences and the extremities of the *trans*-acting ribozyme that may interfere with the binding to its substrate and/or with the proper folding of the catalytically active structure. Following self-cleavage of the transcript (the *cis-trans-cis* cassette), the ribozyme is produced and can interact with its substrate. The ribozyme is protected from cellular ribonucleases by two short and stable stem-loops coming from the *cis*-acting hammerhead motifs.

The aforementioned catalytic properties (i.e. stem length and nucleotide identity) of the hammerhead were also considered for the design of the *trans*-acting ribozyme. The binding of both the ribozyme and substrate give rise to the stems I and III, which are separated by one unpaired nucleotide located adjacent to the cleavage site. Relatively short recognition sequences having a binding energy $\approx$ 15 kcal/mol should favour the stability of the complex without compromising the turnover of the ribozyme (Sioud, 1997). Conversely, long and stable binding sites have the potential to decrease the substrate specificity (i.e. by having a base-pairing other than the desired one) and prevent turnover because the product release may become limiting. Therefore, short recognition sites having a calculated binding energy of $\approx$ 15 kcal/mol were selected. Moreover, stem II was shortened to 5 bp to avoid stem length inhibition coming from the 6 bp stem I. The inactive mutant (G5U) of the ribozyme was also constructed in the same manner.

DNA oligonucleotides corresponding to the hammerhead sequences were annealed and used for cloning (Figure 1B, and for a detailed protocol see the Experimental procedures section). Briefly the *cis*-acting hammerhead duplex (*cis* hh) was cloned into pBluescript II KS (+/–) (*Apa*I and *Sal*I) to yield pRzcis. *Trans*-acting hammerhead (*trans* hh) duplex was then inserted into pRzcis (*Sal*I and *Xba*I). Upon digestion with *Rsr*II, the latter construct produces a *cis-trans* cassette that was cloned into pRzcis to yield the *cis-trans-cis* construct pRz1. In pRz1, the ribozyme (*trans*-acting hammerhead) is surrounded by two *cis*-acting hammerheads allowing the

production of the ribozyme upon self-cleavage of the transcripts. Intermolecular ligation of the *cis-trans* cassette produces multimeric inserts that were used to construct a trimeric form of pRz1. The trimers were then PCR amplified and cloned into plant transformation vectors for the *in vivo* assays (see Experimental procedures). The same approach was used for the cloning of the catalytically inactive mutated ribozyme (pRz1-G5U).

Identification of a potential cleavage site in a target mRNA

Initial experiments were required in order to identify potential cleavage sites within the SPP2 dioxygenase mRNA (accession no. AF104925). In our search for potential hammerhead ribozyme target sequences in SPP2 mRNA, we used three rules: (i) the sequences should harbour the consensus recognition sequence of a hammerhead ribozyme (i.e. 5′-N_5**GUC**/N_6-3′); (ii) these sequences should be located between the initiation codon and the first third of the open reading frame (ORF) in order to minimize the likelihood of retaining an ORF that could still support the production of an active truncated protein; and (iii) the selected sequence should be located in a region that appears to be single-stranded. Target sequences located in single-stranded regions of an mRNA should be more accessible to the ribozyme than those found in double-stranded regions. Within a double-stranded region, the ribozyme might compete unfavourably with intramolecular base-pairing when trying to bind to its substrate (Birikh *et al*., 1997; Campbell and Cech, 1995). Consequently we used the Mfold program (Zuker, 1989), to predict not only the most stable secondary structure (in terms of energy), but also the following nine most stable structures predicted of the complete 1229 nucleotides (nt) mRNA. Regions consistently predicted to be in double-stranded conformation were thus eliminated as potential target sites. The three criteria described above were then applied to these 10 structures. Specifically, we searched for primarily single-stranded regions, which we arbitrarily defined as being more than 10 bases in a 14 nucleotides stretch, and which included the characteristic hammerhead GUC sequence. Two sites were found to respond to these criteria in eight of the 10 structures (named sites -1 and -2, see Figure 2A).

The accessibility of these identified sites was then evaluated using ribonuclease H (RNase H). This enzyme specifically cleaves the RNA moiety of a DNA-RNA duplex, and can be used to verify whether or not an oligonucleotide binds specifically to a target RNA sequence. DNA oligonucleotides (14 nucleotides) corresponding to the recognition domain of the hammerhead ribozyme (site-1 and -2, Figure 2A), and a sequence complementary to positions 110–123 (site-3, negative control, Figure 2A), were synthesized. Site-3 appears to be located in a primarily double-stranded region, regardless of the structure analysed. Radioactively labelled SPP2 mRNA from *in vitro* transcription was pre-incubated with unlabelled oligonucleotides prior to RNase H hydrolysis, and the resulting mixtures resolved on denaturing gels (Figure 2B). Site-1 only showed a moderate level of cleavage (as determined by densitometric analysis), while site-2 exhibited a relatively high level of cleavage of the mRNA by the RNase H, producing both the 1147 and 140 nt products (the 58 nt extra sequence comes from the multiple cloning site of the vector). In both cases, the products from the RNase H hydrolysis, indicates that the reaction occurred at a single site, illustrating the specificity of the DNA oligonucleotide induced cleavage. Finally,

A

Site	Position in mRNA	mRNA sequence oligo sequence	RNase H cleavage
1	91-104	5′ - UCCAAGUCAUUGCA -3′ 3′ - AGGTTCAGTAACGT -5′	+
2	62-75	5′ - AACUGGUCAAGCAC -3′ 3′ - TTGACCAGTTCGTG -5′	+++
3	110-123	5′ - GUGCAUGAAAGACC -3′ 3′ - CACGTACTTTCTGG -5′	–

B

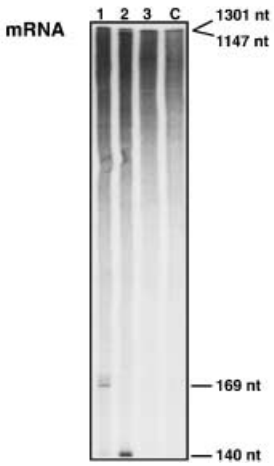

Figure 2 SPP2 mRNA structure probing by RNase H. (A) Summary of the selected sites, the sequences of the mRNA/DNA oligonucleotides, and relative measure of the RNase H cleavage. The numbering refers to the positions within the original cDNA. (B) Autoradiogram of a 5% PAGE gel of RNase H assays. Lanes 1–3 correspond to the sites identified in (A), while lane 4 is a control incubation of the mRNA and RNase H in the absence of an oligonucleotide. The sizes of the RNA species in nucleotides are indicated on the right. An extra 72 nt sequence was added on the transcription product from the cloning procedure.

site-3, as predicted from the secondary structure analysis, appeared to be inaccessible (no detectable cleavage activity observed). In the latter case, if the oligonucleotide and the mRNA were heat denatured and snap-cooled prior to the addition of the RNase H, a treatment which favours the formation of alternative less stable structures, some cleavage could be detected (data not shown). As a control, the *in vitro* transcribed radioactive mRNA was incubated with RNase H alone, without the oligonucleotides. No cleavage product could be detected (Figure 2B, lane 4). These results show that, *in vitro*, site-1 and -2 were accessible, while site 3 was not, as predicted from the secondary structure analysis. Since site-2 seemed to be the most accessible region for the designed of a hammerhead ribozyme, we then determined if such a ribozyme could target both *in vitro* and *in vivo* an endogenous mRNA for degradation.

In vitro transcription of the *cis-trans-cis* cassette

The ribozymes were synthesized in the presence of [α-^{32}P]GTP by *in vitro* transcription of the *Bst*X 1-linearized plasmids using T3 RNA polymerase and aliquots were analysed on 10% polyacrylamide gels (Figure 3A and B). The transcriptions were performed either at 20 °C, which is more representative of normal plant growth conditions, or 37 °C (the optimal reaction temperature of the T3 polymerase). Regardless of the temperature, nearly all full-length transcripts (234 nt band) were processed to give three fragments: an 88 nt fragment containing the *trans*-acting ribozyme, and two other fragments of 53 and 93 nt containing the 5′ and 3′ *cis*-acting ribozymes, respectively. Only trace amounts of transcripts that did not self-cleave at one site (i.e. the 181 and 141 nt bands for the 5′- and 3′-*cis*-acting hammerhead, respectively), were detected. This shows that the level of self-cleavage was not significantly altered by the temperature of incubation. After 120 min of transcription at 37 °C, all *cis*-acting ribozymes have functioned, yielding only the three smaller bands corresponding to the processed *cis*- and *trans*-acting ribozymes (Figure 3B, lane 3). Similar results were observed with a DNA construct producing the inactive version of the *trans*-acting ribozyme (i.e. Rz1-G5U; Figure 3B, lane 4). Moreover, the same extents of self-cleavage were observed when the transcriptions were performed at 20 °C, but a longer incubation time was required (data not shown). The fact that some transcripts were not initially self-cleaved, but were eventually self-cleaved, is noteworthy. This indicates that self-cleavage occurs not only during the elongation phase of the polymerization, but also after the transcription is completed. Thus, engineered *cis*-acting ribozymes with

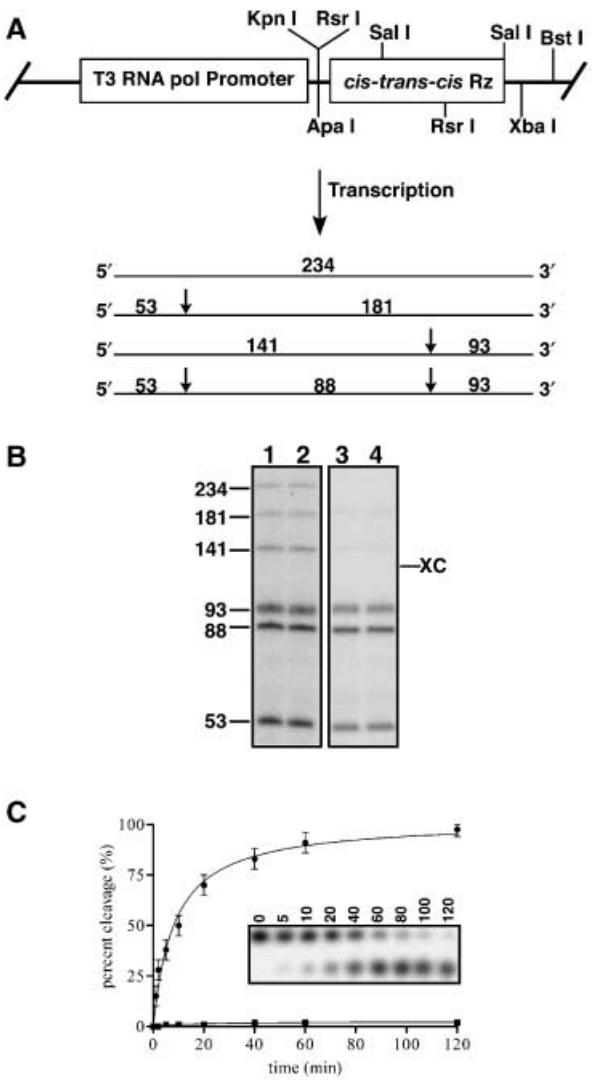

Figure 3 *In vitro* transcription of a *cis-trans-cis* construct. (A) Expected RNA products of self-cleavage during transcription. (B) Autoradiogram of a 5% PAGE gel of *in vitro* transcriptions of the *cis-trans-cis* cassette for either 1 h at 20 °C or 15 min at 37 °C (lanes 1 and 2, respectively). Lanes 3 and 4 represent the transcription products of Rz and Rz-G5U for 2 h at 37 °C, respectively. (C) Time course experiment on the cleavage activity of the ribozymes with a short substrate (16 nt) (upper panel), and a graphical representation of the cleavage percentage as a function of time (lower panel). Circles and squares indicate cleavage catalysed by the Rz1 and Rz1-G5U, respectively.

GNRA tetraloops have the ability to provide only a *trans*-acting ribozyme devoid of long, extra sequences, at least *in vitro*.

Activity of the *trans*-acting ribozyme *in vitro* on its target mRNA and a small model RNA

The *trans*-acting ribozymes produced *in vitro* were extracted from the gel and their activity was determined for cleavage of either a small model RNA, or the full size SPP2 dioxygenase

mRNA under single-turnover conditions ([Rz] >> [S]). The small model substrate (16 nt) possesses three guanosine residues at its 5′-end, which ensures efficient transcription (Milligan *et al*., 1987), followed by the sequence from positions 63 to 75 of the SPP2 dioxygenase mRNA (i.e. 5′-GGGACUG-**GUC**/AAGCAC′-3). Cleavage of a 5′-labelled substrate yields a ^{32}P-labelled-5′-product (10 nt) and a non-radioactive 3′-product (6 nt) (Figure 3C). Only trace amounts of substrate are still detected after 2 h. The cleavage extent of 97.7% indicates that most, if not all ribozyme molecules were active, and demonstrates that the hairpins that remain at both extremities of the transcripts (see Figure 1) do not interfere with the *trans* cleavage. A rate constant of 0.21 min^{-1} was determined, which is in the same order of magnitude as many other efficient ribozyme reported in the literature (Roy *et al*., 1999). Cleavage of the complete mRNA yields products of 148 and 1153 nt (extra sequences come from the cloning vector). We observed a cleavage extent of ≈ 20% when using the complete mRNA (data not shown). The lower yield observed when cleaving the complete mRNA as compared to the small model substrate was expected, since the *trans*-acting ribozyme has to, firstly, bind to the target mRNA and, secondly, induce a conformational change to adopt an active state. This conformational change is expected to be much less efficient when binding a long substrate. Moreover, since the complete mRNA adopts a secondary structure, the overall accessibility is lowered with a more structured molecule. Neither the mRNA nor the small substrate were cleaved by the inactive mutant Rz1-G5U (data not shown), indicating that cleavage by Rz-1 requires ribozyme enzymatic activity. Together these results show that the designed ribozyme is functional *in vitro*.

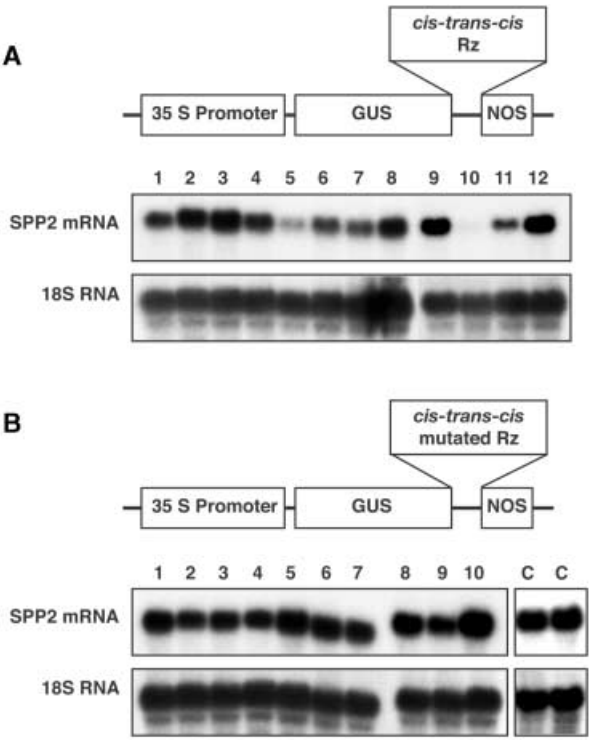

Figure 4 *In vivo* activity of the *trans*-acting ribozyme directed against *Solanum chacoense* dioxygenase mRNA as determined by RNA gel blot analysis. Total leaf RNA (10 μg) from 12 GUS positive transgenic plants harbouring the pBin35GUS-Rz construct (A), and 10 GUS positive transgenic plants harbouring the pBin35GUS-G5U-Rz mutated construct (B) were probed with the 1.2 kb complete (*EcoRI*/*XhoI*) SPP2 cDNA insert. Lower panels for (A) and (B) same as upper panel, except that the membrane was stripped and reprobed with an 18S ribosomal cDNA probe from *S. chacoense*.

Activity of the *trans*-acting ribozyme *in vivo* in transgenic plants

Contrary to the *in vitro* situation, *in planta*, the mRNA will be found associated with numerous mRNA binding proteins that will have an impact on the ribozyme's ability to act on the target RNA. Furthermore, the overall accessibility of the mRNA will be lowered by the adoption of secondary structures. To determine if the designed *cis-trans-cis* ribozyme cassette could be used to target endogenous mRNA *in planta*, the cassettes of pRz1 and pRz1-G5U were subcloned into a plant binary transformation vector and expressed under the control of the CaMV 35S promoter. The targeted SPP2 dioxygenase gene is a single copy gene and showed no cross-hybridization to related dioxygenase under our hybridization conditions (Lantin *et al*., 1999). This ensured that the expression analysis would only monitor one gene, and not a whole gene family. Because the ribozyme construct is small and, furthermore, self-cleaves to liberate even smaller RNA fragments (see Figure 3), the *cis-trans-cis* ribozyme cassette was embedded between a reporter construct, the β-glucuronidase gene (GUS), and the nopaline synthase (NOS) terminator (Figure 4). The resulting constructs, harbouring either the active (pBin35GUS-Rz) or the inactive (pBin35GUS-G5U-Rz) ribozyme were transferred into *Agrobacterium tumefaciens* and used to transform *Solanum chacoense* plants. The transgenic plants expressing the constructs were initially selected based on a simple (non-quantitative) GUS colorimetric assay (data not shown). Since the ribozyme is located just after the GUS ORF within the construct, this ensures that the ribozymes were also expressed to significant levels in the GUS positive selected plants. Twelve GUS positive transgenic plants harbouring the pBin35GUS-Rz construct and 10 GUS positive transgenic plants harbouring the pBin35GUS-G5U-Rz mutated construct were selected for further analyses.

Total RNA was extracted from the leaves of mature transgenic plants and the ribozyme activity on the target SPP2 dioxygenase mRNA was monitored by RNA gel blot analysis (Figure 4). Figure 4A shows that, out of the 12 transgenic plants, six were significantly affected by the ribozyme. Four plants had target mRNA expression levels that corresponded to 36–50% of the wild-type level (plant nos. 1, 6, 7 and 11). In the most severely affected plants (plant nos. 5 and 10), the SPP2 dioxygenase mRNA levels detected corresponded to 15% and 6%, respectively, of unaffected or untransformed control plants. To determine if the effect of the *cis-trans-cis* ribozyme cassette depended strictly on the enzymatic activity of the *trans*-acting ribozyme, or if the reduced level of the target mRNA could be the consequence of RNA silencing through the presence of the two short stems of 6 bp stretches (separated by one nucleotide) that anneal to the target mRNA, transgenic plants harbouring an identical construct, except for a critical nucleotide substitution, were also analysed. The pBin35GUS-G5U-Rz has the same binding ability through an identical complementary region than the pBin35GUS-Rz, but has a G-to-U substitution that renders the ribozyme unable to coordinate a magnesium ion necessary for its enzymatic activity. None of the 10 transgenic plants harbouring the mutated ribozyme construct showed any significant decrease in target mRNA levels (Figure 4B), indicating that, as for the *in vitro* cleavage results (squares in Figure 3C), the ribozyme activity was needed to affect the stability of the targeted mRNA.

Comparison with antisense and sense co-suppressed transgenic lines

In order to compare the efficiency of the ribozyme strategy to reduce target mRNA abundance, we also produced transgenic lines that expressed sense and antisense SPP2 dioxygenase cDNA constructs under the control of the CaMV 35S promoter with doubled enhancer. Plants that regenerated on kanamycin media were randomly selected and first tested for the presence of the transgene (data not shown). Total RNA was extracted from the leaves of mature transgenic plants and the residual mRNA level of the targeted SPP2 dioxygenase was monitored by RNA gel blot analyses (Figure 5). In sense over-expressing lines, it is known that a certain percentage of the plants often show a reduction in target mRNA accumulation, even though over-expression was initially expected, through a post-transcriptional gene silencing mechanism (Fagard and Vaucheret, 2000; Napoli *et al*., 1990). Out of 13 sense over-expressing lines analysed, only two were negatively affected, with one line (line 12) showing almost complete suppression of the targeted gene (Figure 5A). The same analysis was performed for 16 randomly selected antisense lines (Figure 5B). Control expression levels for untransformed plants were as shown in Figure 4B. Since the complete double-stranded SPP2 cDNA was used for probing, a strong accumulation of a band of abnormal size could result from the accumulation of a large amount of antisense strand RNA driven by the strong CaMV 35S promoter. Nonetheless, only one line out of the 16 analysed showed almost complete suppression of the targeted gene (lane 7). Lanes 13 and 15 showed a significant reduction in SPP2 steady-state mRNA levels, while lanes 8 and 16 showed bands of abnormal size, with the expected SPP2 1.2 kb band was absent or largely reduced in abundance (compare with lane 13, which clearly shows these two bands).

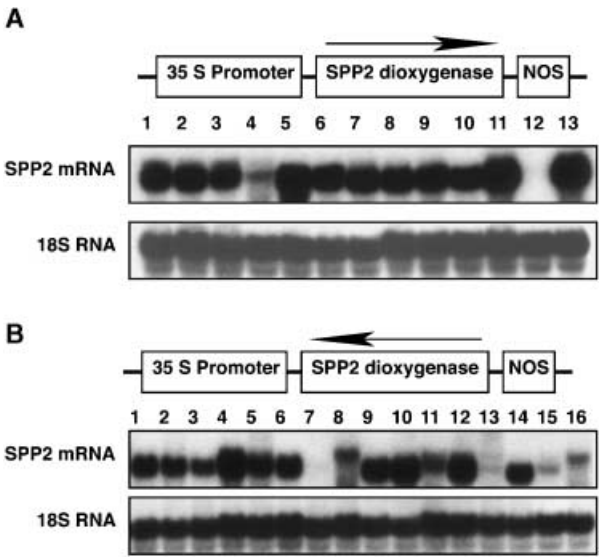

Figure 5 (A) RNA expression analysis of the SPP2 dioxygenase transcript levels in leaves of SPP2 sense transgenic plants. (B) RNA expression analysis of the SPP2 dioxygenase transcript levels in leaves of SPP2 antisense transgenic plants. Total leaf RNA (10 μg) from randomly selected transgenic plants for each constructs were probed with the 1.2 kb complete SPP2 cDNA insert. Lower panels for (A) and (B) same as upper panel except that the membrane was stripped and reprobed with an 18S ribosomal cDNA probe from *S. chacoense* to assure equal loading of all samples.

Discussion

To our knowledge, only a few studies have used ribozymes to down-regulate endogenous mRNAs *in planta*. In the first two cases, the experiments targeting either the potato UDP-glucose pyrophosphorylase (UGPase) or the tobacco anionic lignin-forming peroxidase (TPX), respectively (Borovkov *et al*., 1996; McIntyre *et al*., 1996), did not include as controls

inactive non-catalytic hammerhead sequences, making it impossible to determine if the down-regulation of the target mRNA observed in some transgenic lines was a result of the activity of the ribozyme as a *bona fide* catalytic RNA molecule, or if the action was the result of an antisense or an RNAi effect. In a later attempt, targeted at the stearoyl-ACP Δ9 desaturase in maize (Merlo *et al*., 1998), expression of non-catalytic hammerhead sequences showed that catalytic activity of the ribozyme was indeed necessary to obtain mutant transgenic plants with decreased levels of the desaturase mRNA, although no fragments corresponding to the cleaved mRNA could be detected *in planta*, probably due to a rapid degradation following cleavage. Furthermore, among the plant lines tested, the most severely affected had only a 3.9-fold reduction in target mRNA levels. All three previous experiments used ribozymes that had extra flanking sequences both 5′ and 3′, probably reducing the ability of the molecule to fold properly and interact with the substrate (Bertrand *et al*., 1994; Denman, 1993; Fedor and Uhlenbeck, 1990; Ruiz *et al*., 1997). These sequences included the bar selectable marker gene (Merlo *et al*., 1998) or the GUS gene (Borovkov *et al*., 1996), without the possibility of self-cleaveage for the ribozyme. Thus, the catalytically active RNA was still embedded in a much longer transcript. Similarly to the use of the bar-ribozyme construct (Merlo *et al*., 1998), we used the GUS reporter gene to ensure that our ribozyme constructs were effectively expressed as fusions *in planta* by selecting GUS positive plants. To ensure a maximum likelihood of *trans*-acting activity, we embedded the *trans*-acting ribozyme between two self-cleaving catalytic RNA molecules (Figure 1). Moreover, the molar ratios of the ribozymes *in planta* were increased by the use of multimerized constructs. Using such a *cis-trans-cis* ribozyme cassette, we were able to significantly down-regulate half the transgenic plants tested. Four plants had target mRNA expression levels that corresponded to 36–50% of the wild-type level (plant nos. 1, 6, 7 and 11), while in the two most affected transgenics (plant nos. 5 and 10), the target mRNA level detected corresponded to 15% and 6%, respectively, of unaffected or untransformed control plants. For plant no. 10 this corresponds to a 17-fold reduction in target mRNA levels (Figure 4A). As a control, identical constructs except for a G-to-U mutation that inactivates the catalytic activity but not the complementary region of the ribozyme were also used to transform *S. chacoense* plants. No GUS positive transgenic plants harbouring the mutant ribozyme showed a significant reduction of the target mRNA (Figure 4B), indicating that the down-regulation of SPP2 mRNA levels was due to the catalytic activity of the ribozyme. Although we could not detect the cleavage product of the target SPP2 dioxygenase mRNA, even after long exposures, the activity of the ribozyme was essential to produce plants with reduced levels of SPP2 mRNAs. Once cleaved, the target mRNA were most probably very rapidly degraded. The catalytic activity of our ribozyme construct after self-cleavage from the *cis-trans-cis* cassette was also demonstrated by incubating gel-extracted *in vitro* produced ribozyme mixed with a model 16 nt RNA or with an *in vitro* transcribed target mRNA.

The various *in vitro* transcription experiments performed showed that the designed *cis-trans-cis* cassettes released almost all, if not exclusively, *trans*-acting ribozymes with proper 5′ and 3′ termini, i.e. the *cis*-acting hammerhead motifs exhibit full self-cleavage regardless the temperature of reaction (20 °C or 37 °C). Thus, it seems that the presence of GNRA tetraloops, which were incorporated to promote the folding into the catalytic structure, really enhanced the self-cleavage activity at least *in vitro*. This suggestion receives additional physical support from another study using *cis-trans-cis* cassettes in which the GNRA loops were modified for other nucleotide compositions, as a consequence, the level of self-cleavages were less, down to 50% in some cases (unpublished data, D. Lévesque, K. Fiola and J.-P. Perreault). Clearly, the presence of the GNRA tetraloops is an advantage to this cassette as compared to all others reported previously (e.g. see (Altschuler *et al*., 1992; Feng *et al*., 2001; MacKay *et al*., 1999; Ruiz *et al*., 1997)). The production of proper *trans*-acting hammerhead ribozymes *in planta* remains to be shown but we have good reasons to believe that it was also efficient because *in vitro*, even at 20 °C, self-cleavage was near completion. More importantly, in order to cleave the target mRNA, colocalization is necessary. Since the selected plants showed GUS enzymatic activity, this indicates that the chimeric GUS-ribozyme mRNA was localized in the cytoplasm. Although self-cleavage reactions of the *cis-trans-cis* cassette could occur immediately after transcription in the nucleus, the reduced cleavage rate observed at 20 °C would ensure that most of the GUS-ribozyme transcripts have reached the cytoplasmic compartment, and that the *cis*-cleavage of the flanking ribozyme has released the *trans*-acting ribozyme *in vivo* in the cytoplasmic compartment.

The overall efficiency of the procedure was also tested against two other widely used strategies to down-regulate the expression of selected genes. A similar number of both sense and antisense over-expressing lines were produced using the full length SPP2 dioxygenase target gene under the control of the same promoter as for the ribozyme-expressing transgenic lines. Although a similar number of almost fully suppressed lines were obtained in all three cases, only the

ribozyme-mediated strategy produced a higher number of plants expressing intermediate levels of the targeted gene. Since the ribozyme is acting directly on the target messenger RNA as an enzyme, this probably reflects the expression levels of the ribozyme constructs in individual transgenic lines. This wider range of expression levels is also an advantage when analysing phenotypes, enabling the monitoring of a more subtle effect instead of an all or nothing effect.

Recently, RNA silencing through the expression of hairpin RNA molecules transcribed *in planta* has been successfully used to down-regulate the expression levels of multiple genes (Chuang and Meyerowitz, 2000; Smith *et al*., 2000; Waterhouse *et al*., 1998). This strategy has gained favour with many plant biologists because of its ease of use and higher specificity than antisense mediated post-transcriptional gene silencing (O'Brien *et al*., 2002), although mismaches can be tolerated in the 21 nt duplex RNA unless they are located in the middle of the siRNA (Elbashir *et al*., 2001). Furthermore, the use of long sense and antisense hairpin constructs increases the chance of cross-reactions with closely related genes, or in multigene families. The fact that ultra-short hybridizing arms were used in our ribozymes substantially decreased the likelihood of cleavage of any other RNA molecules. This also increases the turnover rate, and stability is provided by the secondary structure of the ribozyme and the GNRA tetraloops. Temperature is another important aspect where the use of ribozyme can be beneficial if not absolutely necessary. Low temperatures have been shown to inhibit RNA silencing by the control of siRNA generation (Szittya *et al*., 2003). In this case, the authors examined the reasons behind the fact that outbreaks of virus disease in plants are frequently associated with low temperatures. They were able to show that both virus- and transgene-triggered RNA silencing were inhibited at low temperatures. What is remarkable here is that even at a normal growth temperatures, the level of siRNA produced was substantially lowered. As an example, in virus-induced silencing, siRNAs were undetectable at 15 °C, and barely detectable at 21 °C. Since no protein complex is necessary for ribozyme-mediated transcript inactivation, and since we were able to demonstrate strong cleavage activity for plants kept at a constant temperature of 20 °C, this would suggest that ribozyme-mediated transcript inactivation has other advantages over gene silencing.

Currently, the potential of using ribozymes in plants is limited to only a few examples producing crops resistant to either viroids or viruses. This report constitutes an original demonstration that ribozymes may also be used to target an endogenous mRNA in plants. More importantly, this work shows that it is possible to engineer a very efficient *cis* cassette for the expression of ribozymes which is free of undesirable sequences, with very high specificity and independent of a protein-based processing step.

Acknowledgements

We thank Dr David Baulcombe (John Innes Centre, UK) for pointing out the pertinent literature related to RNA silencing. We also thank Gabriel Téodorescu for plant care and maintenance. This work was sponsored by grants from the Natural Sciences and Engineering Research Council of Canada to D.P.M. and J.P.P. S.L. and F.B. were the recipients of PhD fellowships from the Fonds pour la Formation de Chercheurs et l'aide à la recherche (FCAR-FQRNT). J.P.P. is an investigator of Canadian Institutes for Health Research, and D.P.M. holds a Canada Research Chair in Functional Genomics and Plant Signal Transduction.

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