Silencing Genes Involved in Post-transcriptional Gene Expression

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Introduction

Genes involved in RNA metabolism have key roles in post-transcriptional mRNA processing. We are in the process of developing protocols that express these genes transiently and will allow us to study the various roles of these genes in plants. Here we describe the use of a microRNA to reduce transcription of tobacco RNA guanylyl transferase and an Sm-Like protein.

Silencing with short dsRNAs

MicroRNAs are short regulatory RNAs that are processed from double stranded RNA to direct cleavage of target endogenous mRNAs. miRNA39/171 is a developmentally regulated miRNA complementary to scarecrow-like transcription factors. The primary miRNA sequence is 123nt long with a 46nt structured loop region (Fig 1). The 21nt sequence complemetary to scarecrow has a 2nt mismatch in the primary sequence, but the processed 21nt miRNA is perfectly complementary to its target sequence.

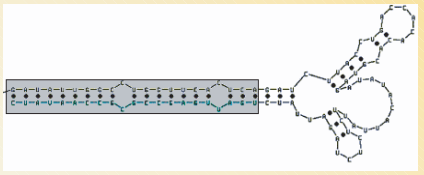

Fig.1 Primary miRNA39/171 and secondary structure.
Sequence complementary to scarecrow is highlighted.
This region was substituted for sequence complementary to GFP, StRGT and NtLSm4.

To determine whether the short 46nt loop region could substitute for an intron sequence in a dsRNA construct, we replaced the 1347nt *Petunia hybrida* Chalcone synthase A intron with the 46nt miRNA39/171 loop sequence and also reduced the length of the dsRNA sequence from 630nt to 100nt (Fig 2A). Co-agroinoculation of a GFP expressing construct with ds630GFPint or ds100GFPmiR led to a loss of GFP expression (Fig 2B) and RT-PCR analysis of RNA isolated from agroinoculated leaf discs confirmed the loss of GFP transcripts in the ds630GFPint and ds100GFPmiR samples (Fig2C). This data shows that the shorter miRNA loop sequence is as effective at inhibiting GFP expression as dsRNA constructs containing intron sequence which has been shown to improve silencing by dsRNA constructs.

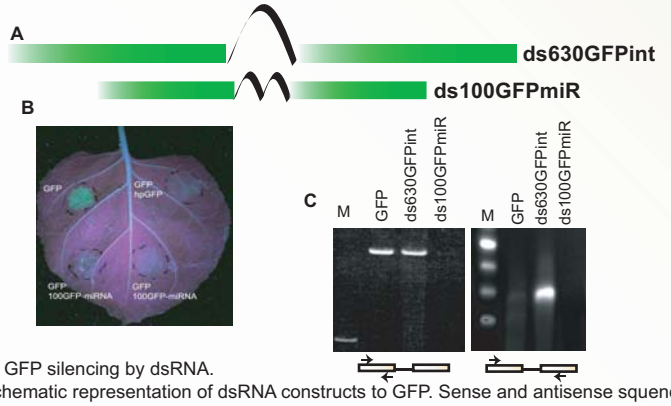

Fig.2 GFP silencing by dsRNA.
A. Schematic representation of dsRNA constructs to GFP. Sense and antisense squences are separated by either the *CHS A* intron or the miRNA39/171 loop sequence. Green box, GFP sequence, B. Co-agroinoculation of GFP with constructs that contain ds630GFPint, ds100GFPmiR and empty vector control. C. RT-PCR analysis of co-agroinoculation leaf discs to show expected 138nt GFP product.

We further reduced the length of the complementary sequence to 21nt to create 3 different siRNA constructs targeted to different parts of the GFP coding sequence (ds21a,b and c GFPmiR, Fig3A). In an attempt to improve the short dsRNA sequence as a substrate for processing to the miRNA, we extended the length of the stem and included the 2 mismatches to recreate the stem sequence found in the original miRNA39/171 (ds21eGFPmiR) (Fig 3A). In all cases there was a poor reduction in GFP expression after co-agroinoculation with GFP (Fig 3B). However after analysis of GFP transcripts in agroinoculated leaf discs, the levels of GFP transcripts were found to be reduced (Fig 3C).

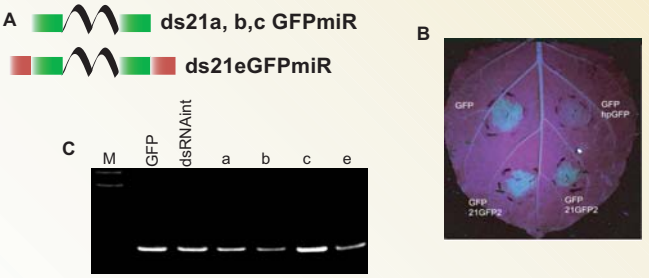

Fig.3 GFP silencing by short dsRNA.
a. Schematic representation of short dsRNA constructs to GFP. Green box, GFP sequence, Red boxes original miRNA39/171 stem sequence. b. Co-agroinoculation of GFP with constructs that contain ds630GFPint, ds21bGFPmiR and empty vector control. c. RT-PCR analysis of co-agroinoculation leaf discs to show expected 138nt GFP product.

Silencing plant post-transcriptional factors

As part of a general strategy to investigate the effects of reducing key genes involved in various plant post-transcriptional processes, we used the short dsRNAs to reduce expression of RNA guanylyl transferase (RGT) and an Sm-Like protein (LSm4). RGT catalyses the condensation of GTP with the 5' end of pre-mRNAs to form the CAP. The CAP has many essential roles in RNA metabolism by providing resistance to endonucleases, participating in pre-mRNA splicing, mRNA 3' end formation, mRNA transport and translation. LSm4 is one of seven Sm-like proteins that form a heptomeric ring around Sm binding sequences and has roles in pre-mRNA splicing and mRNA degradation.

Silencing of RGT and LSm4 transcription was compared using short dsRNA constructs (ds21a,b,c RGTmiR and ds21a,b,c LSm4miR, Fig4A). Silencing constructs were agroinoculated into leaves of *Nicotiana benthamiana* and then changes in RGT and LSm4 transcript levels were assayed by RT-PCR. In two out of the three short RGTmiR constructs (b and c), RT-PCR analysis showed reduced levels of target mRNA. Similarly in LSm4, all three short dsRNAmiR constructs led to a reduction in target transcripts (Fig 4B). Ubiquitin levels remained constant. Similar experiments with dsRNAint and co-suppression to LSm4 showed similar reductions in transcrpt levels compared to the control (data not shown).

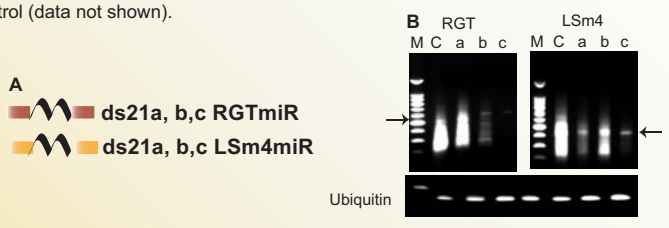

Fig.4 Agroinoculation of short dsRNAs to RGT and LSm4 in tobacco leaves
A. Schematic representation of ds21RGT and LSM4miR. B. RT-PCR analysis of RGT and LSm4 transcripts from tobacco leaf discs isolated after agroinoculation with three ds21miR constructs, a,b and c. Ubiquitin was used as a control to measure constant mRNA levels.

To confirm the knockdown of RGT and LSm4 by short dsRNAs, constructs were transfected into mesophyll protplasts from *Nicotiana tabacum*. As a control for cell viability, protoplasts were also transfected with a full length sense plant SR protein (SC35) (A. Barta, Vienna, Austria), which has previously been shown to lead to protoplast death. The integrety of protoplasts was assayed by fluorescein diacetate and counting viable protoplasts compared to the total number of protoplasts. Visually, there was little difference between the water control and the short dsRNAs (Fig5A). However, cell counts showed that transfection of the RGT and LSm4 target RNAs led to a 20% reduction in viable protoplasts (Fig 5B). Only 8% viable protoplasts were found after overexpression with the control SC35. Using equal amounts of RNA in all cases, RT-PCR analysis showed a reduction of both RGT and LSm4 while the levels of ubiquitin remained constant (Fig 5C).

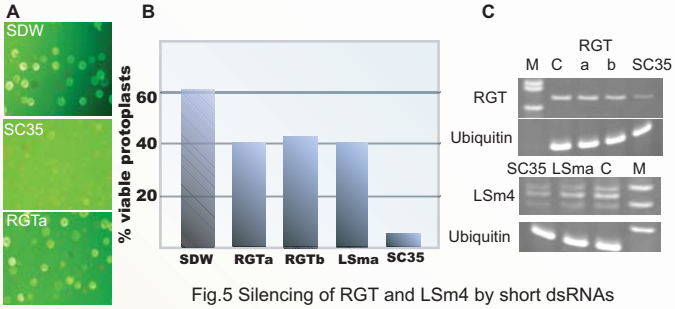

Fig.5 Silencing of RGT and LSm4 by short dsRNAs in tobacco protoplasts
A. Protoplasts stained with Fluorescein.
B. Protoplasts viability count.
C. RT-PCR analysis of protoplast RNA

Summary

- miRNA39/171 loop substitutes for the *CHS* A intron in dsRNA constructs.
- 100nt dsRNA silences GFP strongly, while 21 dsRNA silences weakly.
- 21dsRNAs also silences RGT and LSm4 weakly in both leaves and in protoplasts

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