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Characterization of the P0 gene of *Sugarcane yellow leaf virus*

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Contents

List of Abbreviations

AGO	Argonaute
cDNA	complementary DNA
dpi	days post infiltration
dsRNA	double-stranded RNA
HC-Pro	helper component-proteinase
miRNA	micro RNA
nt	nucleotide
PCR	Polymerase Chain Reaction
PTGS	posttranscriptional gene silencing
qRT-PCR	quantitative Real-Time-PCR
RDR	RNA dependant RNA polymerase
rgs-CaM	regulator of gene silencing- calmodulin-like-protein
RISC	RNA induced silencing complex
SCYLV	*Sugarcane Yellow Leaf Virus*
sGFP	sense GFP
siRNA	small interfering RNA
Skp1	S-phase kinase related protein
ta-siRNA	trans-acting small interfering RNA
WEL1	Werner exonuclease 1
YLS	Yellow Leaf Syndrome

1. Introduction

1.1 Viral Suppression of PTGS

Posttranscriptional gene silencing (PTGS) in plants is an evolutionarily conserved mechanism in which short (21-24 nt) RNAs mediate the degradation of homologous RNA transcripts in a sequence specific way. Although there are several PTGS pathways, they all share common core reactions that are triggered by double-stranded (ds) RNA in the cytoplasm. In sense-transgene-mediated silencing this key component is generated by the RNA-dependant RNA polymerase RDR6. Improperly terminated, unpolyadenylated aberrant mRNA serves as template for the RNA polymerase and dsRNA is formed (Gazzani et al. 2004; Luo et al. 2007). By contrast, inverted-repeat-encoded dsRNAs, antisense transgenes capable of forming duplexes with target gene transcripts, and viral RNAs, which depend on viral RNA-dependant RNA polymerase, enter the PTGS pathway directly at the dsRNA step (Meins et al. 2005). dsRNAs are cleaved by an RNaseIII enzyme of the Dicer family into short RNA duplexes; one strand becomes incorporated as an siRNA into an Argonaute containing multicomponent complex known as RISC (RNA-induced silencing complex). The siRNA guides the complex to complementary mRNA and mediates transcript cleavage. (Baulcombe, 2004; Voinnet, 2005; Pazhouhandeh et al. 2006). In addition to cell-autonomous silencing, plants also generate a mobile signal at the site of silencing initiation, after which the signal spreads systemically and induces specific gene suppression in distant parts of the plant (Voinnet and Baulcombe. 1997).
PTGS is important in host defense against viruses, and it is believed that many plant viruses encode silencing suppressor proteins, which can overcome this defense response. These proteins probably evolved independently in different virus groups, because they are structurally diverse, and they do not contain common sequence motifs (Baulcombe. 2004).
One method for suppression of silencing involves suppressor proteins that bind sRNAs, a strategy exemplified by the tombusviral p19 and poleroviral p21 proteins. It has been shown that p19 specifically binds *in vitro* to synthetic and natural ds siRNAs having 2-nt 3’ overhangs. Lakatos et al (2004) have demonstrated that p19 inhibits

RNA silencing *in planta* by binding and sequestering silencing-generated siRNAs, whereby RISC activation is inhibited. Crystal structure analysis of p19 – siRNA complexes have revealed that a p19 homodimer forms a rectangular two layered structure from which two α-helices project at opposite ends of the homodimer, thereby measuring and bracketing both ends of the siRNA duplex. Consequently, an siRNA or miRNA is prevented from being incorporated into an active RISC (Ye et al 2003).

In addition to p19, p21 appears to prevent silencing by directly binding siRNA. Ye and Patel (2005) reported the crystal structure of p21, which reveals an octameric ring structure with a large central cavity. They proposed a model where 21 nt or longer single and double stranded RNAs interact with the conserved, positive charged inner surface of the ring.

The recruitment of endogenous negative regulators of PTGS represents a further silencing-suppression strategy. Anandalakshmi et al (2000) proposed a model where suppression of PGTS by the helper component-proteinase (HC-Pro) of plant potyviruses is mediated by the activation of rgs-CaM (regulator of gene silencing-calmodulin-like-protein). *N. benthamiana* plants overexpressing rgs-CaM resembled plants expressing the potyviral HC-Pro suppressor of gene silencing, indicating that rgs-CaM acts as a suppressor of PTGS. Their findings highlight the probability of rgs-CaM being an endogenous mediator of silencing suppression in a calcium dependent pathway.

A third strategy is illustrated by AC2, a geminivirus transcriptional-activator protein, modifying the host transcriptome. AC2 possesses three conserved domains: a basic domain with a nuclear localization signal at the N terminus, a central DNA-binding domain with a nonclassical Zn-finger motif, and an acidic activator domain at the C terminus. Mutation analysis of those domains suggested that silencing suppression is mediated at the host DNA level. RNA profiling experiments revealed a set of genes whose transcripts are highly induced in the presence of AC2. Among those were six cold-regulated genes, which could be adaptive for the virus given that RNA silencing is inhibited by low temperatures. Moreover, WEL1 (Werner exonuclease 1), a known positive regulator of RNA silencing *in C. elegans* and *A. thaliana*, was upregulated. Transient overexpression experiments in N. benthamiana showed that WEL1 is capable of suppressing RNA silencing, indicating that WEL1 exerts a dominant-negative effect (Trinks et al. 2004).

1.2. *Sugarcane yellow leaf virus*

Sugarcane yellow leaf virus (SCYLV) is the causal agent of sugarcane Yellow Leaf Syndrome (YLS). The disease pattern is characterized by bright yellowing of the abaxial surface of the midrib on the four or five youngest leaves, which spreads throughout the leaf tissue followed by necrosis from the leaf tip to the base. Roots and stalks show impaired growth, and production is significantly reduced. Vega et al. (1997) have demonstrated that SCYLV is restricted to the cytoplasm of phloem companion cells. Virion particles from YLS-diseased plants are transmitted to disease-free plants by the sugarcane aphid, *Melanahis sacchari*, the corn leaf aphid, *Rhopalosiphum maidis*, and the rice root virus aphid, *R. rufiabdominalis* (Schenck et al. 2000).

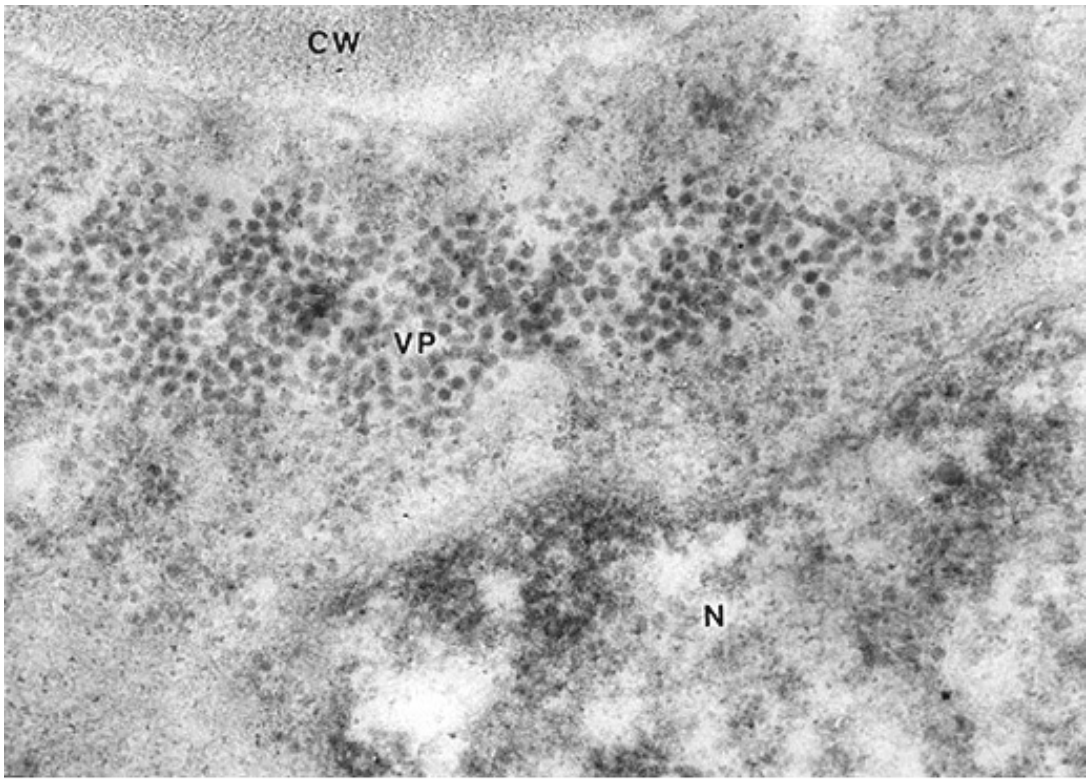


Figure 1: In infected sugarcane plants virus particles aggregate in the cytoplasm of phloem companion cells. VP, virus particles; CW, cell wall; N, nucleus. Source: Vega et al. (1997)

SCYLY is a member of the Luteoviridae family, and has emerged through recombination between a *Polervirus*, a *Luteovirus*, and an *Enamovirus* (Moonan et al 2000). The 5' part of the SCYLV genome is of *Polerovirus* origin and the first open reading frame is termed P0.

1.3. Function of the P0 proteins

The deduced amino acid sequence of P0 proteins among different geographic isolates is highly conserved. Interestingly, the amino acid sequence of SCYLV P0 ($P0^{SC}$) to P0 proteins from different *Polerovirus* species is relatively low, with $P0^{PL}$ (P0 potato leaf roll virus) displaying the highest similarity of 21% (Mangwende et al. unpublished data). Despite this apparent diversity, $P0^{SC}$, $P0^{PL}$, $P0^{BW}$ (*Beet western yellow virus*), and $P0^{CA}$ (*Cucurbit aphid-borne yellow virus*) are all suppressors of PTGS (Peffer et al. 2002).

$P0^{BW}$ and $P0^{CA}$ possess an F-box like domain, whose integrity is essential to mediate silencing suppressor activity (Pazhouhandeh et al.2006). The F-box is a loose concensus sequence of about 70 amino acids required for the interaction between a given F-box protein and Skp1 (S-phase kinase related protein 1), a subunit of the E3 ubiquitin ligase complex of the SCF subfamily (Hermand 2006).

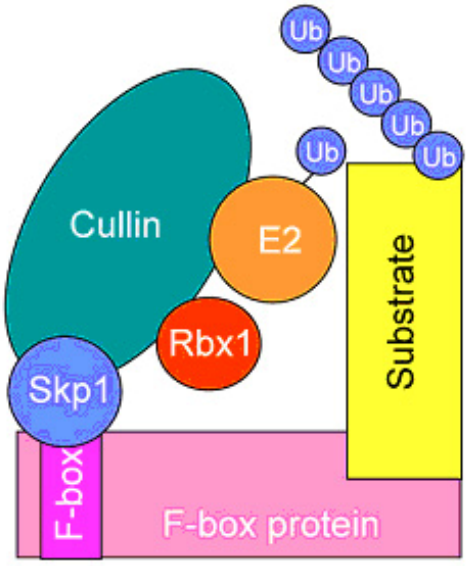


Figure 2: Schematic depiction of the SCF ubiquitin ligase complex. An F-box protein and its linked substrate bind via the F-box and Skp1 interaction to the main complex, which mediates ubiquitination of the substrate. Source Hermand (2006)

Pazhouhandeh et al (2006) have demonstrated that $P0^{BW}$ and $P0^{CA}$ interact with ASK1 and ASK2, the Arabidopsis homologs of SKP1. This prompted the idea that P0 proteins together with ASK1/2, RBX1, and Cullin would form an SCF-like E3 ubiquitin ligase involved in the specific ubiquitylation of a silencing related protein, thereby targeting it for proteasome degradation. It was later shown that $P0^{BW}$ and $P0^{CA}$ interact with Argonaute1 (AGO1) *in planta* and *in vivo*, thereby provoking decay of the labelled AGO1 proteins, supporting the previously mentioned theory (Bortolamiol et al. 2007). To deliver insight into the proposed mode of AGO1 degradation by the proteasome, $P0^{BW}$ and AGO1 were transiently co-expressed while proteasome activity was inhibited. However, PO still had a destabilizing effect on AGO1. This observation indicates that there may be alternative models of a P0 mechanism, including the possibility of a degradation mechanism that may not involve ubiqiuitin or proteasomes. Further research has revealed that the presence of a PAZ and an adjacent ND domain of AGO1 are necessary for P0 mediated decay of AGO1 (Baumberger et al. 2007). Due to the participation of AGO1 in secondary siRNA generation, this might explain the huge drop of secondary siRNA level – in contrast to

an abundant level of primary siRNA – in the presence of $P0^{BW}$ and $P0^{CA}$. Taken together, these findings suggest a silencing suppressor mode downstream of Dicer function in PTGS, where P0 proteins act via their F-boxes with ASK1/2 to degrade Argonaute proteins in a not yet described way (Baumberger et al. 2007; Bortolamiol et al. 2007).

Similar to the previously mentioned P0 proteins, PO^{SC} contains an F-box like domain. In addition to a suppressor function in local PTGS, $P0^{SC}$ is capable of suppressing systemic PTGS and inducing cell death in infiltrated areas of *N. benthamiana* leaves (Mangwende et al. unpublished data). Since AGO1 belongs to a multigene family comprised of 10 members in *A. thaliana*, these novel activities inspired us to investigate if any further Agonaute protein is targeted by $P0^{SC}$.

1.4. Structure and Function of Argonaute proteins

The objects of this study were AGO1 and AGO10, its closest relative. Additionally, AGO4 and AGO7 were chosen to represent distant clades of this multigene family.

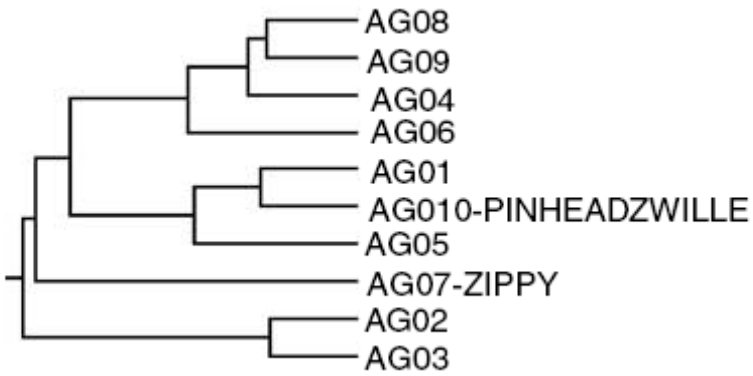


Figure 3: Phylogenetic tree of the *Arabidopsis* Argonaute proteins. Source: Zheng et al. (2007)

Briefly, Argonaute proteins harbour four distinct domains: N-terminal, PAZ, Mid, and PIWI. The PAZ domain binds to siRNA and miRNA and enables an Argonaute to recognize the 3' overhang of a siRNA/miRNA, thus permitting it to distinguish between siRNA/miRNA and degraded RNA. The PIWI domain has an RNase-H like fold and possesses endonucleatic activity (Hutvagner and Simard. 2008). AGO1 is a RNA slicer that selectively recruits sRNAs. It is associated with miRNAs, endogenous ta-siRNAs, transgene derived siRNAs (Baumberger and Baulcombe.2005). AGO4 controls locus specific siRNA accumulation and DNA methylation (Zheng et al. 2007). AGO7 does not have a specific role in transgene silencing; its main purpose is to regulate development via the ta-siRNA pathway (Hunter et al. 2003; Adenot et al. 2006). AGO10 is engaged in meristem maintenance, but was not found to participate in PTGS (Moussian 1998).

1.5. Overview

The aim of this project was to determine if PO^{SC} targets Argonaute proteins for degradation; thus revealing similaritries and differences to other P0 proteins, which would give us additional insight into silencing suppressor strategies. After the candidate genes had been chosen, we had to frame a detection method for the targets in subsequent western blots. AGO4, AGO7, AGO10 were tagged with FLAG sequences at the N-terminus, a strategy which had been already successfully demonstrated for AGO1. Since Dr. David Baulcombe provided us with *A. thaliana* plants expressing FLAG AGO1, we decided to design an *A. thaliana* line expressing PO^{SC}, which could be used in coexpression experiments. PO^{SC} mediated destruction of AGO4, AGO7, and AGO10 was studied by *Agrobacterium* leaf infiltration in *N. benthamiana* 16C.

2. Materials and Methods

2.1. Plasmid Design

As previously described, several sets of plasmids had to be designed for subsequent *A. thaliana* transformations and *N. benthamiana* in planta transient co-expression experiments.

The blueprint for all plasmid designs was to amplify the target gene cDNA by PCR, separate the products by gel electrophoresis, cut out the corresponding bands, and purify the products (QIAquick GEL Purification Kit).

All the PCR amplifications for expression constructs were performed with Phusion DNA polymerase kit (Finnzymes). $P0^{SC}$ was amplified using primers with 5' *Bgl*II and 3' *Sal*I restriction sites; AGO4 and AGO7 were amplified using primers with 5' *Sal*I restriction sites and a FLAG sequence provided with a start codon (Met* Asp Tyr Lys Asp Asp Asp Asp Lys) and 3' primers, that did not introduce any new restriction sites. AGO10 was amplified with a 5' primer, which was similar to the previously described Argonaute forward primers, and a 3' primer bearing an *Xho*I restriction site.

The purified fragments were cloned into the vector pCR8/GW/TOPO (Invitrogen).

The orientation of the inserts was confirmed by restriction digests and afterwards, $P0^{SC}$ was cloned into pMDC30 and pMDC32 by performing LR recombination reactions (Invitrogen Gateway LR Clonase II Enzyme Mix), while only pMDC32 FLAG AGO4 and pMDC32 FLAG AGO7 were constructed.

During the construction process FLAG AGO10 fragments tended to integrate in reverse orientation; therefore, a 'classical' approach was chosen. FLAG AGO10 was cloned from *Sal*I-*Xho*I digests of pCR8 into corresponding sites of pENTR1A (Invitrogen), which served as an alternative entry vector for LR recombination reactions with pMDC32.

All constructs were confirmed correct by sequencing (University of Hawaii, Center for Genomics, Proteomics, and Bioinformatics Research Initiative).

Finally, *A. tumefaciens* C58 C1 (Ti plasmid pCH32) was transformed with the relevant plasmids using the freeze-thaw method (Hofgen and Willmitzer 1988). The

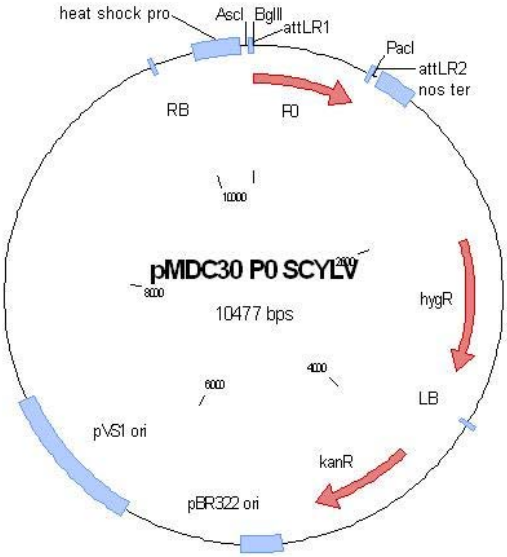
presence of the constructs in C58 C1 was analyzed by colony PCR or by PCR, where isolated plasmid DNA (Fermentas GeneJET Plasmid Miniprep Kit) of the transformed *A. tumefaciens* strains served as template.

The plasmids pMDC30 (heat-shock promoter) and pMDC32 (2x 35S promoter) were chosen as destination vectors, because they are compatible to the Gateway system and are a widely used tool for inducible and constitutive gene expression (Curtis and Grossniklaus 2003).

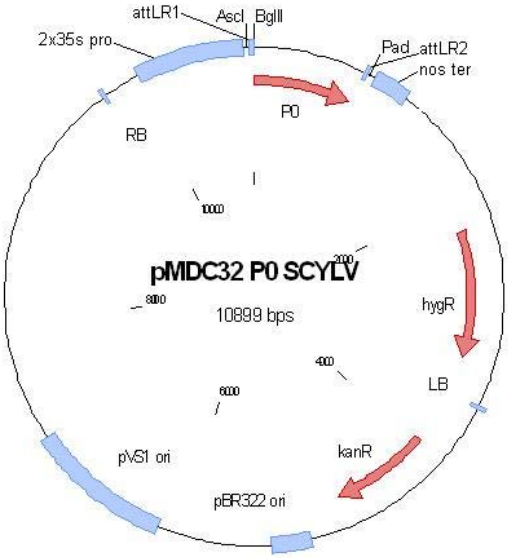
$P0^{SC}$	
BglII P0 F	5' AGATCTATGCTTTTCAACGAATTC 3'
P0 SalI R	CGTAGTCGACCTATATATCATGAGAATAGGTG
Argonaute 4	
CTA SalI FLAG GTC AGO4 F	5' CTAGTCGACATGGACTACAAGGACGACGATGA CAAAGTCATGGATTCAACAAATGGTAAC 3'
AGO4 R	GTACTTAACAGAAGAACATGGAGTTG
Argonaute 7	
CTA SalI FLAG GTC AGO7 F	5' TAGTCGACATGGACTACAAGGACGACGATGAC AAAGTCATGGAAGAAAAAACTCATCATC 3'
AGO7 R	5' GTCATCAGCAGTAAAACATGAGATTC 3'
Argonaute 10	
CTA SalI FLAG GTC AGO10 F	5'CTAGTCGACATGGACTACAAGGACGACGATGAC AAAGTCATGCCGATTAGGCAAATGAA 3'
AGO10 XhoI R	5' TACCTCGAGTTAGCAGTAGAACATTACTCTCTTCA 3'

Table 1: Primers used for expression constructs

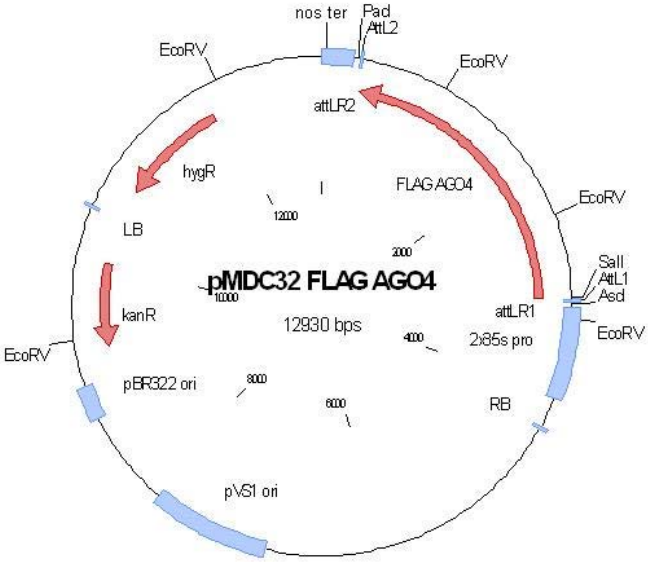
A. pMDC30 P0SCYLV



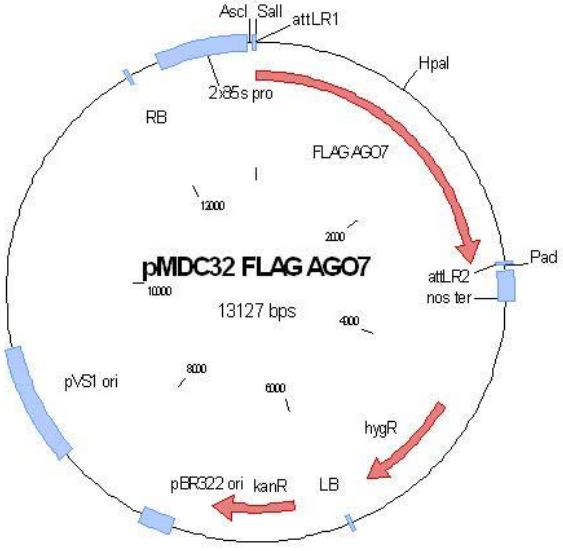
B. pMDC32 P0SCYLV



C. pMDC32 FLAG AGO4



D. pMDC32 FLAG AGO7



E. pMDC32 FLAG AGO10

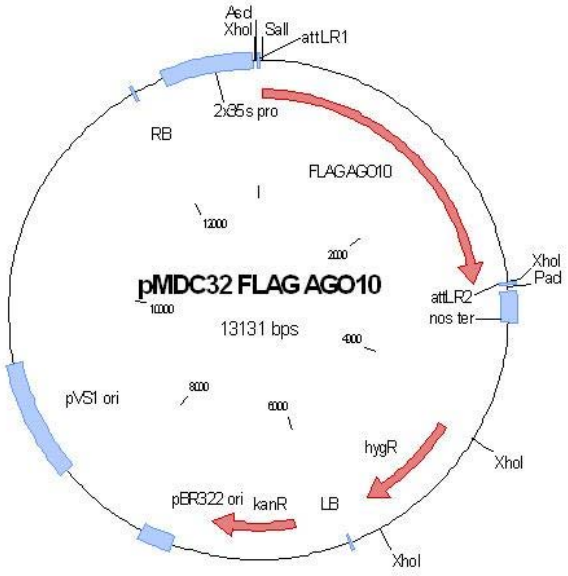


Figure 4: Maps of designed expression constructs. kanR, kanamycin resistance; hygR, hygromycin resistance; nos ter, nos terminator; pBR322 ori, pBR322 origin; 2x35S pro, 2x35S promoter; heat shock pro, heat-shock promoter; L/RB, left/right border

2.1.1. Amplification of target gene cDNA

P0SC and *A. thaliana* Argonaute genes were amplified using high-fidelity Phusion polymerase per manufacturers protocol (Finnzymes). Each amplification was set up as a 20 µl reaction containing each primer at 0.6 µM and 1 ng of template DNA.

A. thaliana Argonaute 7 (NCBI accession number AY394564) cDNA clone was obtained as a generous gift from Dr. Poethig (Hunter et al. 2003). *A. thaliana* Argonaute 10/Zwille (NCBI accession number AJ223508) cDNA clone was a generous gift of Dr. Laux (Moussian et al. 1998). To ensure amplification of full length cDNA, extension times and annealing temperatures were individually adjusted to fit recommendations. During final extension all samples were incubated with *Taq* DNA polymerase. *Taq* DNA Polymerase catalyzes nontemplate addition of a single deoxyadenine to the 3' end of an amplification product (Smith et al. 1995) and allows cloning into the linearized vector pCR8 provided with 3' deoxythymidine overhangs.

Cycling Conditions	$P0^{SC}$	FLAG AGO4	FLAG AGO7	FLAG AGO10
1x Initial denaturation	98°C 30 s	98°C 30 s	98°C 30 s	98°C 30 s
40x Denaturation Annealing Extension	98°C 10 s 54°C 20 s 72°C 30 s	98°C 10 s 56°C 20 s 72°C 90 s	98°C 10 s 56°C 20 s 72°C 90 s	98°C 10 s 58°C 20 s 72°C 90 s
1x Final Extension (Incubation with *Taq* Polymerase)	72°C 10 min	72°C 10 min	72°C 10 min	72°C 10 min

Table 2: Cycling conditions

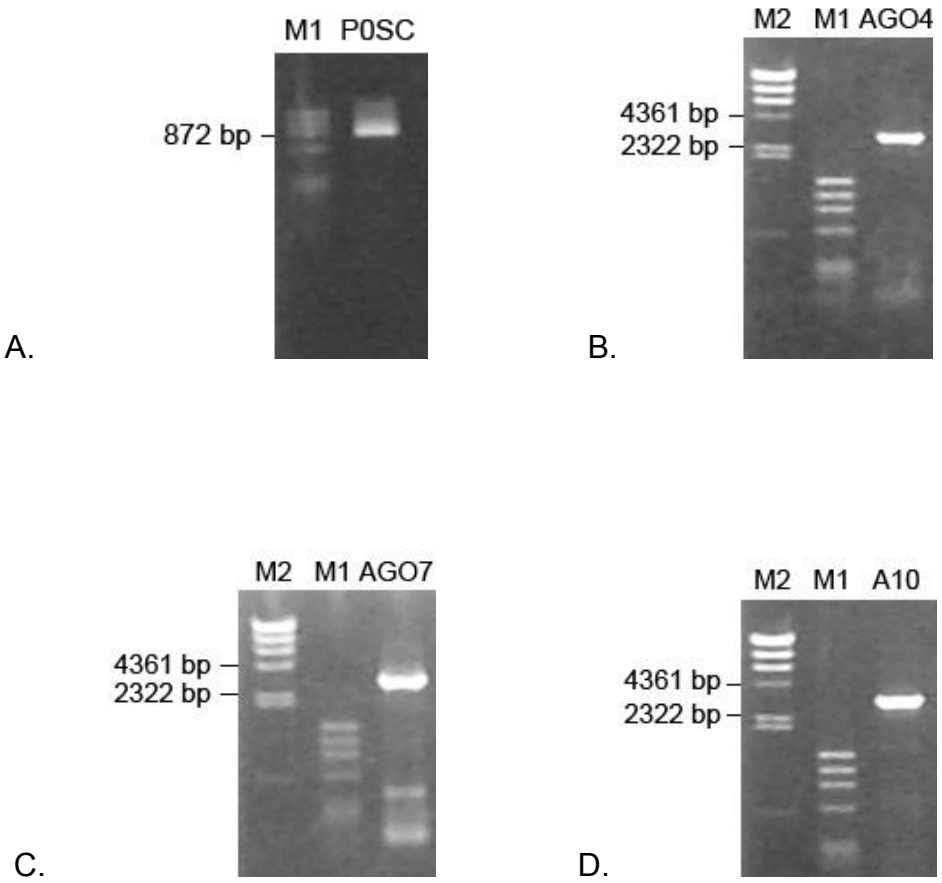


Figure 5: Amplified full length cDNA fragments analyzed on 1% agarose gels. A. P0SC, $P0^{SC}$ (calculated length: 787 bp); B. AGO4, FLAG AGO4 (calculated length: 2818 bp); C. AGO7, FLAG AGO7 (calculated length: 3015 bp), D. A10, FLAG AGO10 (calculated length: 3015 bp)
M1, marker ΦX174 *Hae*III digest; M2, marker Lambda DNA-*Hind*III digest

2.1.2. Design of FLAG AGO10 construct

Restriction digests revealed that FLAG AGO10 fragments amplified with the originally designed primers CTA SalI FLAG GTC AGO10 F and AGO10r (5' GTCATTAGCAGTAGAACATTACTC 3') tended to integrate in reverse orientation when cloned into the TOPO vector pCR8. To overcome this problem, the reverse primer AGO10 XhoI R was designed and in turn PCR products were cloned into pCR8.

pCR8 FLAG AGO10	insert 5'→3'		insert 3'→5'	
diagnostic enzyme combination	*Sal*I *Xba*I	*Hind*III *Eco*RV	*Sal*I *Xba*I	*Hind*III *Eco*RV
expected fragments (bp)	2573 3242	2885 1794 1136	5571 244	3701 1794 320

Table 3: Restriction digests of pCR8 FLAG AGO10

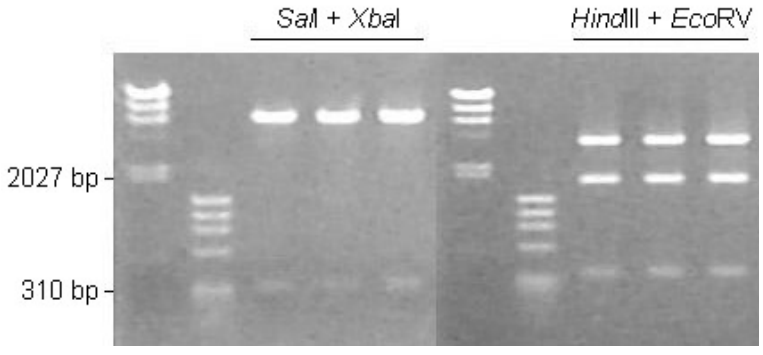


Figure 6: Restriction analysis of pCR8 FLAG AGO10 constructs. FLAG AGO10 fragments were amplified with the primers CTA SalI FLAG GTC AGO10 F and AGO10r. Restriction analysis showed that all constructs contained the insert in reverse orientation.

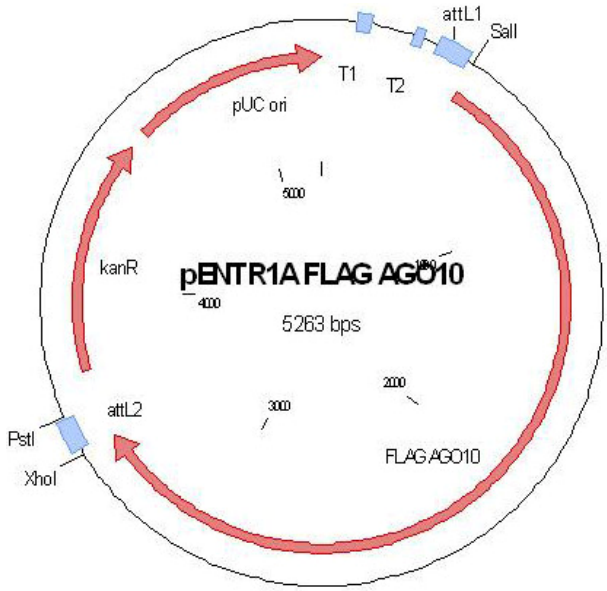
Restrictions of pCR8 FLAG AGO10 and the vector pENTR1A were set up as 20 µl reactions containing 1 µg and 0.5 µg plasmid DNA and the restrictions enzymes *Sal*I and *Xho*I at the concentration of 1unit per 1 µg DNA. Restricted plasmid DNA was analyzed electrophoretically on 1% agarose gels, fragments corresponding to FLAG AGO10 and the open vector were cut out, and gel purified.

Ligations were set up as 10 µl reactions containing insert and vector at a ratio of 7:1 and 1 unit T4 DNA ligase. Ligation reactions were kept at 16°C overnight. *E. coli* TOP10 cells were transformed with the plasmid pENTR1A FLAG AGO10 and orientation of the inserts were confirmed by restriction analysis after prior plasmid isolations.

pENTR1A FLAG AGO10		
diagnostic enzyme combination	*Sal*I *Pst*I	*Xho*I *Pst*I
expected fragments (bp)	3129 2134	5137 126

Table 4: Restriction digests of pENTR1A FLAG AGO10

A.



B.

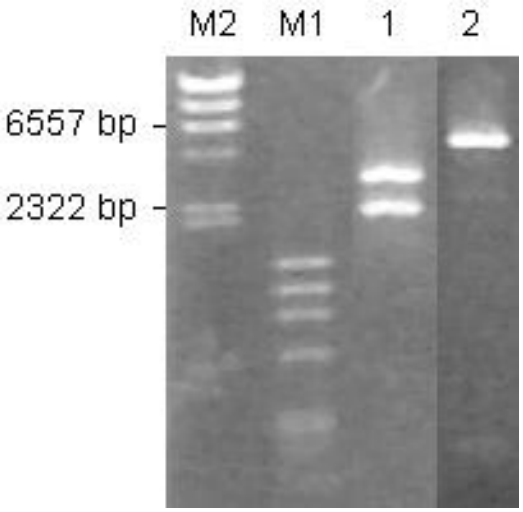


Figure 7: A. Map of pENTR1A FLAG AGO10. pUC ori, pUC origin; kanR, kanamycin resistance; T1/2, *rrn*B T1/2 transcription termination sequence B. Restriction analysis of pENTR1A FLAG Ago10. 1, Digest with *Sal*I and *Pst*I; 2, Digest with *Xho*I and *Pst*I; M1, marker ΦX174 *Hae*III digest; M2, marker Lambda DNA-*Hind*III digest

2.1.3. Restriction digests of plasmids

Restriction digests were conducted to confirm orientation of inserts. Each restriction was set up as a 20 µl reaction containing 0.2 – 0.4 µg plasmid DNA and combinations of restrictions enzymes at the concentration of 1unit per 1 µg DNA. Restricted plasmid DNA was analyzed electrophoretically on 1% agarose gels.

plasmid	pMDC30 P0SCYLV	pMDC32 P0SCYLV	pMDC32 FLAG AGO4
diagnostic enzyme combination	*Acc*65I *Sal*I	*Acc*65I *Sal*I	*Hind*III
expected fragments (bp)	9281 848 348	10039 848	10144 2786

plasmid	pMDC32 FLAG AGO7			pMDC32 FLAG AGO10	
diagnostic enzyme combination	*Pst*I *Sal*I	*Nsi*I *Sal*I	*Bam*HI	*Sal*I *Xho*I	*Hind*III *Pac*I
expected fragments (bp)	12282 845	6131 3766 1754 1476	10138 1698 759 532	7587 3003 1382 1094	9205 2861 1065

Table 5: Restriction digests of pMDC30 POSCYLV, pMDC32 P0SCYLV, pMDC32 FLAG AGO4/7/10

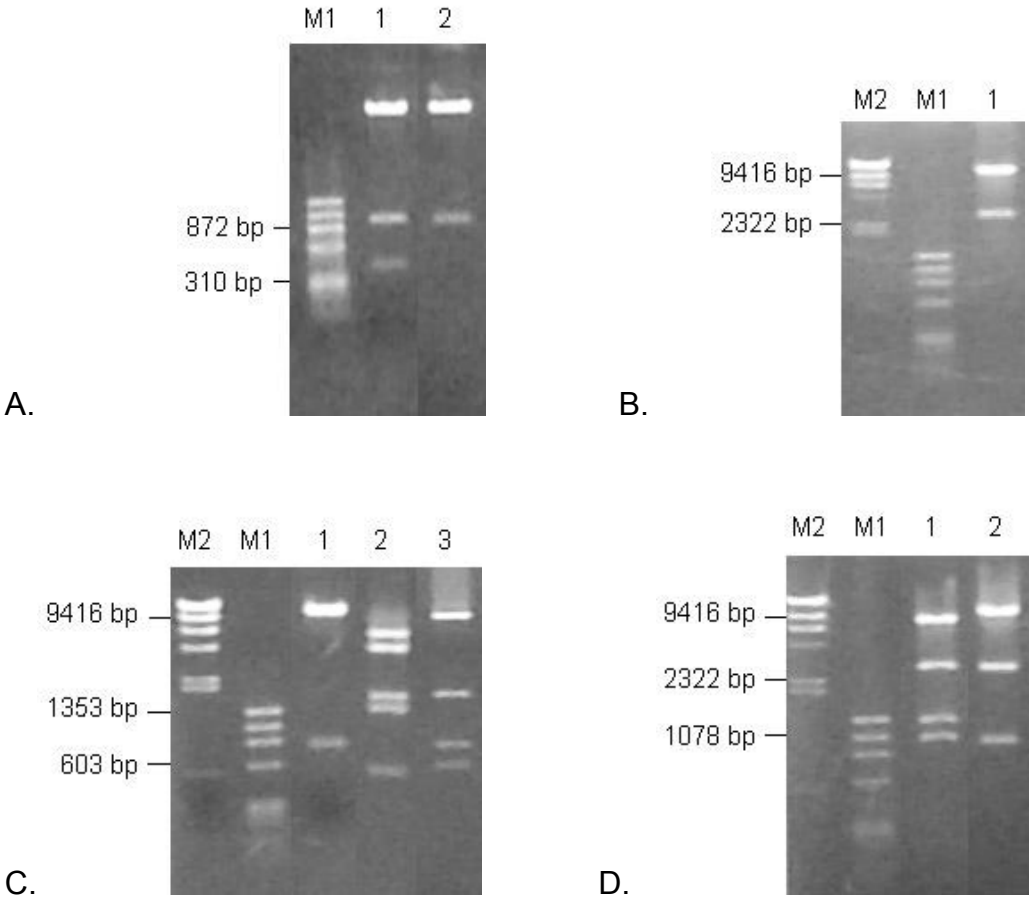


Figure 8: Restriction analysis of designed constructs A. 1, Digest of pMDC30 P0 SCYLV with *Acc*65I and *Sal*I; 2, Digest of pMDC32 P0SCYLV with *Acc*65I and *Sal*I. B. 1, Digest of pMDC32 FLAG AGO4 with *Hind*III. C. 1, Digest of pMDC32 FLAG AGO7 with *Pst*I and *Sal*I; 2, Digest of pMDC32 FLAG AGO7 with *Nsi*I and *Sal*I; 3, Digest of pMDC32 FLAG AGO7 with *Bam*HI. D. 1, Digest of pMDC32 FLAG AGO10 with *Sal*I and *Xho*I; 2, Digest of pMDC32 FLAG AGO10 with *Hind*III and *Pac*I.
M1, marker ΦX174 *Hae*III digest; M2, marker Lambda DNA-*Hind*III digest

2.1.4. *E. coli* transformation

Chemical competent cells (25 µl) were transformed with 1 µl plasmid DNA and incubated on ice for 30 minutes. Cells were heat-shocked by incubation at 42°C for 30 seconds in a water bath. Heat-shock treatment was followed by addition of 200 µl S.O.C. medium and incubation at 37°C for one hour. 20 µl and 100 µl of each transformation were spread on selective plates.

2.1.5. *A. tumefaciens* transformation

A. tumefaciens C58 C1 (Ti plasmid pCH32) was transformed with the relevant plasmids using the freeze-thaw method (Hofgen and Willmitzer 1988). 150 µl of *A. tumefaciens* cells were thawed on ice and up to 10 µl plasmid DNA (total amount: 1 µg) were added. Cell suspensions were gently mixed and frozen in liquid nitrogen for five minutes. Frozen samples were thawed at room temperature and 1 ml LB medium was added followed by incubation at 28°C for four hours with shaking. 50 µl and 100 µl of each transformation were spread on selective plates.

2.2. *A. thaliana* experiments

2.2.1. *A. thaliana* growth conditions

Seeds were kept at 4°C for two days before sowing. Arabidopsis plants were grown at 22°C under 16 hours of illumination.

2.2.2. *A. thaliana* Transformations

Two sets of six week old *A. thaliana* Col-0 plants were transformed by floral dip (Weigel and Glazebrook 2006) with pMDC30 P0 and pMDC32 P0. After three weeks, dried seeds were harvested, sterilized and spread on selective medium.
Due to heavy contamination only two putative pMDC30 P0 transgenic lines could be isolated.
The primary transformants were grown until maturity and the seeds of this self cross were harvested, sterilized and spread on selective medium again. After two weeks a 3:1 ratio was observed and the plants presumably harboring the construct were isolated and grown on separate plates. Three weeks post germination the plants were transplanted into autoclaved soil.

Arabidopsis selection medium
MS-medium
23 g MS-Salts
150 g sucrose
500 mg myo-inositol
2.5 g MES
5 ml Thiamine
Add approximately 4.8 l H_2O.
Set to pH 5.7.
Adjust volume to 5 l with H_2O.
Add 3 g gelrite (AgriBio) per liter.

Selection-media
Cefotaxime: Add 10 ml H_2O to 2 g lyophilized Cefotaxime salts.
Add 625 µl per 500 ml.
Hygromycin: conc.: 20 µl/ml

2.2.3. Heat shock induction

As recommended by Curtis and Grossniklaus (2003) six pMDC30 P0 plants of the T_1 generation were heat shocked in a incubator for 16 hours at 37°C. As a control, six additional pMDC30 P0 plants were kept at 22°C under standard growth conditions. Afterwards, leaves and flower buds were separately harvested and immediately frozen in liquid nitrogen and stored at – 80°C for subsequent RNA isolations.

2.2.4. Total RNA isolation

Previously harvested plant tissue was ground in liquid nitrogen to a fine powder. Total RNA was isolated using the *mir*Vana™ miRNA Isolation (Ambion) kit. 20 µl total RNA per sample were DNaseI (DNA-Free Ambion) treated and the RNA concentration was measured by spectrophotometry.

2.2.5. Quantification of $P0^{SC}$ mRNA

200 ng of DNaseI treated sample RNA was converted into cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems) per manufacturer's protocol. 4 µl of this reaction served as template DNA in Quantitative Real-Time-PCR reactions. As an internal standard the ACT2 (arabidopsis gene i.d. AT3G18780) was chosen, one of the best known and most frequently used reference transcripts for qRT-PCR in plants and animals (Czechowski et al. 2005). Primers were designed to produce amplicons of 193 bp (ACT2) and 165 bp ($P0^{SC}$) with calculated Tm values of 61°C.

$P0^{SC}$	
P0 q2f	5'AAGCACTTCACCCGTCTTTC 3'
P0 q2r	5' GCGTGTGAGTGGATCTTGATAG 3'
Actin	
ACT2fRT-PCR	5' ATCACGATTGGTGCTGAGAG 3'
ACT2rRT-PCR	5' GGTCAGCGATACCTGAGAAC 3'

Table 6: Primers used for qRT-PCR

2.3. *N. benthamiana* experiments

2.3.1. *N. benthamiana* growth conditions

The GFP transgenic N. benthamiana line 16c (Riuz et al. 1998) was grown at 25°C under 12 hours of illumination for four weeks prior to *Agrobacterium* leaf infiltrations.

2.3.2. *N. benthamiana* leaf infiltrations

According to Llave et al. (2000) single *A. tumefaciens* C58C1 colonies harboring a relevant binary plasmid were grown for two days in 5 ml cultures (LB, 100 µg/ml rifampicin, 12.5 µg/ml tetracycline) at 28°C. This was used to inoculate 50 ml cultures (LB, 20 µM acetosyringone, 10 mM Mes/ pH 5.7, 12.5 µg/ml tetracycline). The cultures were incubated for 16 hours at 28°C. Agrobacteria were pelleted at 5000 rpm for 10 minutes and resuspended to an OD_{600} of 0.5 in infiltration medium (10 mM MgCl2, 10 mM Mes/pH 5.7, 150 µM acetosyringone) and incubated at room temperature for 3 hours before infiltration. For a given experiment, different bacterial suspensions of the same optical density and volume were mixed and used.

For *Agrobacterium* leaf infiltrations in *N. benthamiana* pGD (Goodin et al 2002) binary plasmids containing $P0^{SC}$ and $P0^{CA}$ were used instead of the previously described pMDC32 P0SCYLV construct.

Ten plants were infiltrated per treatment. Three days post infiltration plants were illuminated with two 100W ultraviolet (365 nm) lamps and GFP fluorescence in the infiltrated areas were photographically documented.

2.3.3. Total RNA isolation

The infiltrated leaves were harvested three days post infiltration and immediately frozen in liquid nitrogen. The tissue was ground with pre-chilled mortar and pestle, under liquid nitrogen, to a fine powder and stored at – 80°C. Total RNA was isolated using the *mir*Vana^{TM} miRNA Isolation (Ambion) kit. 20 µl raw RNA per sample were DNaseI (DNA-Free Ambion) treated and the RNA concentration was measured by spectrophotometry.

2.3.4. Quantification of Argonaute mRNA

Four hundred nanograms DNaseI treated RNA was converted into cDNA using the TaqMan Reverse Transcription Reagents (Applied Biosystems) in a 20 µl reaction. After synthesis, cDNA was diluted four fold with water and 4 µl of this diluted cDNA served as template for each quantitative RT- PCR. Each amplification was set up as a 25 µl reaction containing each primer at 300 nM and 1X Platinum SYBR Green qPCR Supermix UDG (Invitrogen). *N. benthamiana* actin (GenBank CN747386) was used as endogenous standard to quantify the different Argonaute transcripts. Primers were designed that produce amplicons of 119 bp (actin), 175 bp (FLAG Ago4), 199 bp (FLAG Ago7), and 159 bp (FLAG Ago10). Real-time PCR was performed with a thermal profile of 50°C, 2 min; 95°C, 2 min, 40 amplification cycles of 95°C, 15 sec; 58°C, 20 sec; 72°C, 30 sec; followed by the default denaturation protocol to determine amplification product Tm values.Fluorescence threshold was set at 0.2 relative light units. Fold-change in steady state amounts of Argonaute mRNAs, normalized to actin and relative to empty vector treatment, was calculated as:

$$(E_{AGO}^{CT\ ref\ AGO}/E_{actin}^{CT\ ref\ actin})/(E_{AG0}^{CT\ sample\ AGO}/E_{actin}^{CT\ sample\ actin})$$

Actin	
ACT2f RT-PCR	ATCACGATTGGTGCTGAGAG
ACT2r RT-PCR	GGTCAGCGATACCTGAGAAC
FLAG AGO4	
AGO4f RT-PCR	CTCACGCTGGAATGATTGG
AGO4r RT-PCR	GCTGCCAAGTGAGCATAG
FLAG AGO7	
AGO7f RT-PCR	GATTGTAGCCCTTCAGTAGC
AGO7r RT-PCR	CACCGTCCCTGAAGAATATG
FLAG AGO10	
AGO10f RT-PCR	TCACCACACTCGTTTGTTTG
AGO10r RT-PCR	CTGCTTGTTCCCTGAATACC

Table 6: Primers used for qRT-PCR

2.3.5. Protein extraction

In the course of the experiments, several protein extraction buffers were used and tested. In the end, protein extractions were performed as previously described by Bortolamiol et al (2007). The concentration of protein was measured by Bradford protein assay to ensure equal loading of protein at ensuing gel electrophoresises. Additionally, equal loading was controlled by coomassie brilliant blue staining of a replica gel. Protein extracts were analyzed by migration on precast NuPAGE Novex 8% Bis Tris gels using the NuPAGE electrophoresis system (Invitrogen) according to the manufacturer's recommendations, and transferred onto Immobilon-P membranes (Millipore). Proteins were immunodetected with monoclonal ANTI-FLAG M2 antibody (Sigma) in conjugation with alkaline phosphatase anti-mouse IgG (H+L) (Vector Laboratories) using the WesternBreeze Chemiiluminescent Western Blot Immunodetection kit (Invitrogen).

Buffers

Extraction buffer I

120-150 mg fresh plant tissue

+ 600 µl extraction buffer

PBS: 137 mM NaCl

10 mM Na_2HPO_4

2 mM KH_2PO_4

pH = 7.4

+ 0.01% NaN_3

prior to use add: 1g PVP-40 / 50 ml

65 mg Na_2SO_3 / 50 ml

40 µl proteinase inhibitor (25 x) ('complete' from Roche)

0.1 % Tween 20 ($C_{58}H_{114}O_{26}$)

Protocol

- Cool on ice 10 min
- Homogenize
- Cool on ice 1 min
- Repeat homogenization
- Cool on ice 5 min
- Centrifuge 14k x rpm in cold room 20 min
- Collect supernatant and respin as above
- Collect supernatant ~300 µl / sample and store at – 80°C

Extraction buffer II

20 mM Tris pH=7.5
300 mM NaCl
5 mM $MgCl_2$
1.5 mM DTT
5% PVPP sigma P-6755
1% PVP
0.5 % NP40
~2x proteinase inhibitor

Protocol

- Add 3 ml/g extraction buffer per sample.
- Cool on ice for 10 minutes.
- Vortex.
- Spin down (16 000 g / 4°C).
- Transfer supernatant into fresh tube.
- Spin again.

2.3.6. Dot blot

Dot Blots were conducted to assure the functional integrity of ANTI-FLAG M2 antibody. Therefore, N-Terminal Met-FLAG-BAP control protein was denatured for 15 minutes at 95°C. Afterwards, it was kept on ice to prevent the renaturation of bacterial alkaline phosphatase (BAP), which would interfere with the BAP of the secondary antibody. 1 ng, 3 ng, 5 ng, and 10 ng of the denatured control protein were spotted on a membrane and immunodetected the same way as the samples from the protein extraction. As a control, denatured N-Terminal Met-FLAG-BAP control protein was incubated only with chemilumininescent substrate to confer the non-renaturation of the BAP.

2.4. DNase treatment protocol

Kit: DNA-Free by Ambion

20 µl raw RNA
2.5 µl 10 x Buffer
1 µl rDNase 1

- Vortex.
- Incubate at 37°C for 30 min.
- Add an additional µl rDNase 1.
- Incubate at 37°C for 30 min.
- Add 5µl DNase inactivation reagent.
- Vortex.
- Spin down.
- Move liquid (leave behind white precipitate) to new tube.

2.5. cDNA Production protocol

Kit:
TaqMan Reverse Transcription Reagents
Applied Biosystem by Roche Molecular Systems, Inc

Protocol:

RNase free water	5.7 µl
10x TaqMan Buffer	2.0 µl
25 mM MgCl2	4.4 µl

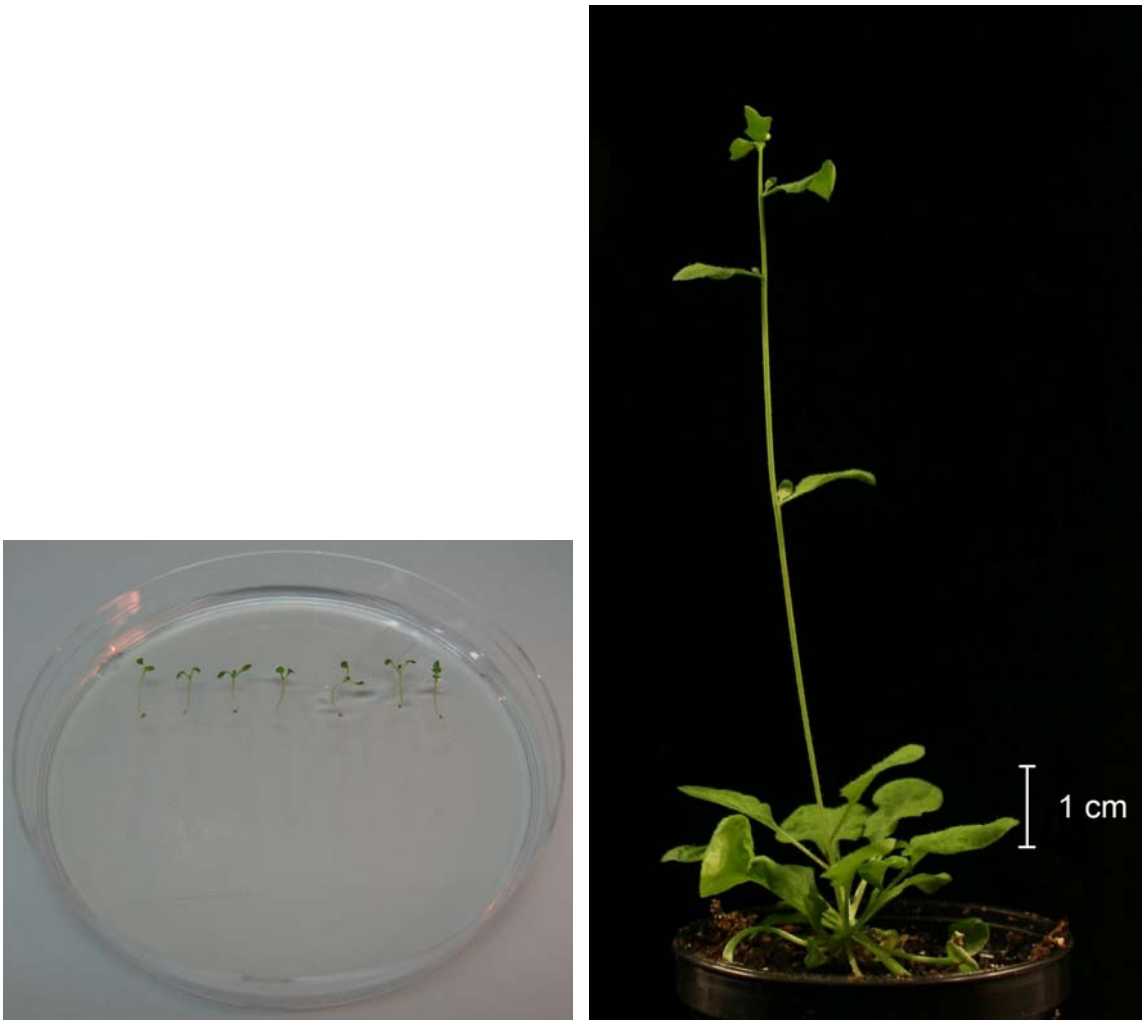
dNTP mix (2.5 mM)	4.0 µl
oligo dT/Random Hexamer	1.0 µl
RNase inhibitor	0.4 µl
Reverse Transcriptase	0.5 µl
RNA (200 ng/µl)	2.0 µl

Primer annealing step	25°C / 10 min
RT reaction	48°C / 30-60 min (up to 2 hours)
RT inactivation	95°C / 5 min

3. Results

3.1. *A. thaliana* experiments

If $P0^{SC}$ was the mediator of Argonaute protein degradation, it would be possible to observe this prediction in coexpression experiments. To test this assumption, two constructs harboring $P0^{SC}$ under the control of a constitutive or an inducible promoter were designed and transformed into *A. thaliana* Col-0 plants, which could be crossed into provided FLAG AGO1 plants. Due to heavy contamination and the proposed ability of $P0^{SC}$ to mediate cell death, only two primary transformants of $P0^{SC}$ under the control of a heat shock promoter were isolated. The primary transformants were grown until maturity and the seeds of this self-cross were harvested, sterilized and spread on selective medium again. Only one line was able to reach T_1 stage, because a breakdown of the air-condition system destroyed our efforts and so we had to mourn the loss of one putative transgenic line. Line #2 was grown until it reached maturity and total RNA from leaves and buds was isolated after heat shock induction. In the meantime, primers were designed to measure transgene expression of $P0^{SC}$ by qRT-PCR. Lack of time was the hindering obstacle to examine transgene expression, which would have given us insight, if this putative transgenic line was a powerful tool for coexpression experiments.



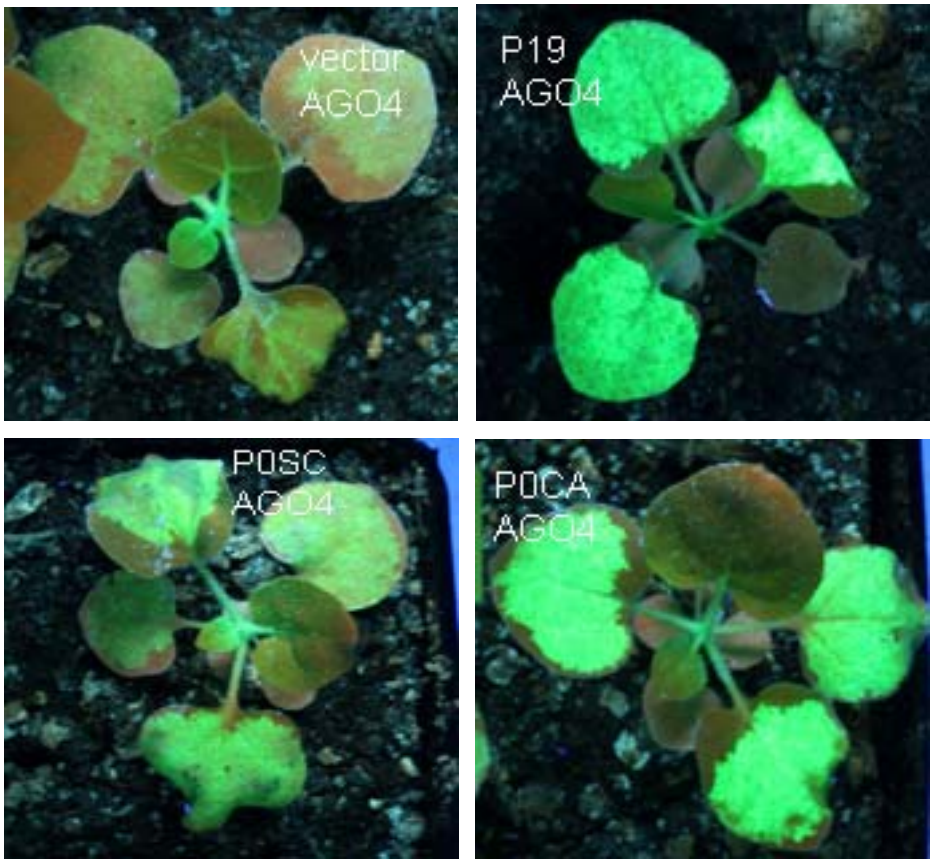
A. B.

Figure 9: A. 2 week old T_1 seedlings of pMDC30 P0 transformants (line #2) grown on MS Hyg^{20} medium. B. Adult T1 pMDC30 P0 transformants prior heat shock induction.

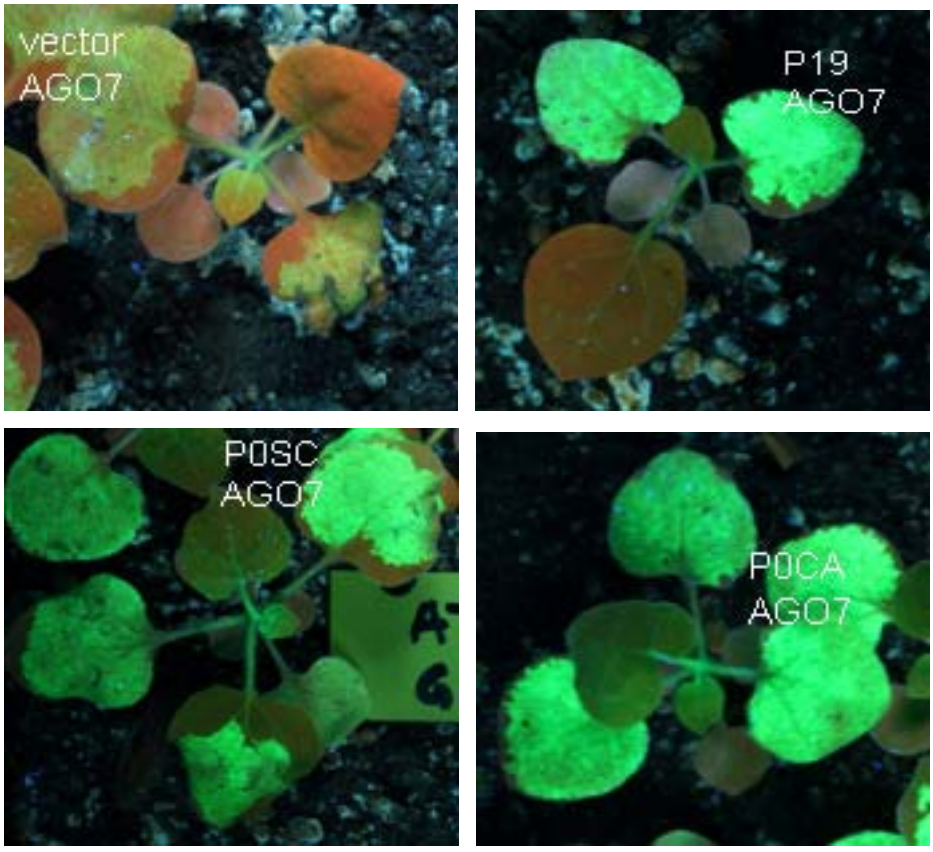
3.2. *N. benthamiana* leaf infiltrations

$P0^{SC}$ mediated degradation of Argonaute proteins was studied by agrobacterium leaf infiltration in 16c *N. benthamiana* (Johansen and Carrington 2001; Llave et al. 2000). *N. benthamiana* leaves were infiltrated with three *A. tumefaciens* C58C1 cultures of the same optical densities mixed in equal proportions. Three culture infiltrations consisted of following components: one of three tagged Argonaute constructs, a silencing suppressor, and sGFP. sGFP was used to ensure and monitor coexpression of the tested suppressors, since an active suppressor would prevent PTGS of sGFP. An empty binary vector served as negative control. The p19 gene of *Tomato bushy stunt virus* (Dunoyer et al. 2004; Takeda et al. 2002) was used as positive control suppressor. $P0^{CA}$ (Pazhouhandeh et al. 2006) was used to draw comparisons between $P0^{SC}$ and P0 proteins from different species. Three days post treatment leaves infiltrated with the different AGO constructs and sGFP together with the empty vector showed signs of PTGS. Infiltrations of the different AGO constructs and sGFP plus p19 showed bright green fluorescence throughout the infiltrated area. Triple infiltrations of AGO, sGFP with $P0^{SC}$ or $P0^{CA}$ showed green fluorescence of similar intensity. In contrast to $P0^{CA}$, leaves infiltrated with $P0^{SC}$ tended to wrinkle and signs of cell death were observed. Although cell death was not the object of this work and was not monitored separately over a longer period, this additional finding is consistent with previous results (Mangwende et al. unpublished data).

A. 3 dpi, 16c, infiltrated with sGFP +



B. 3 dpi, 16c, infiltrated with sGFP +



C. 3 dpi, 16c, infiltrated with sGFP +

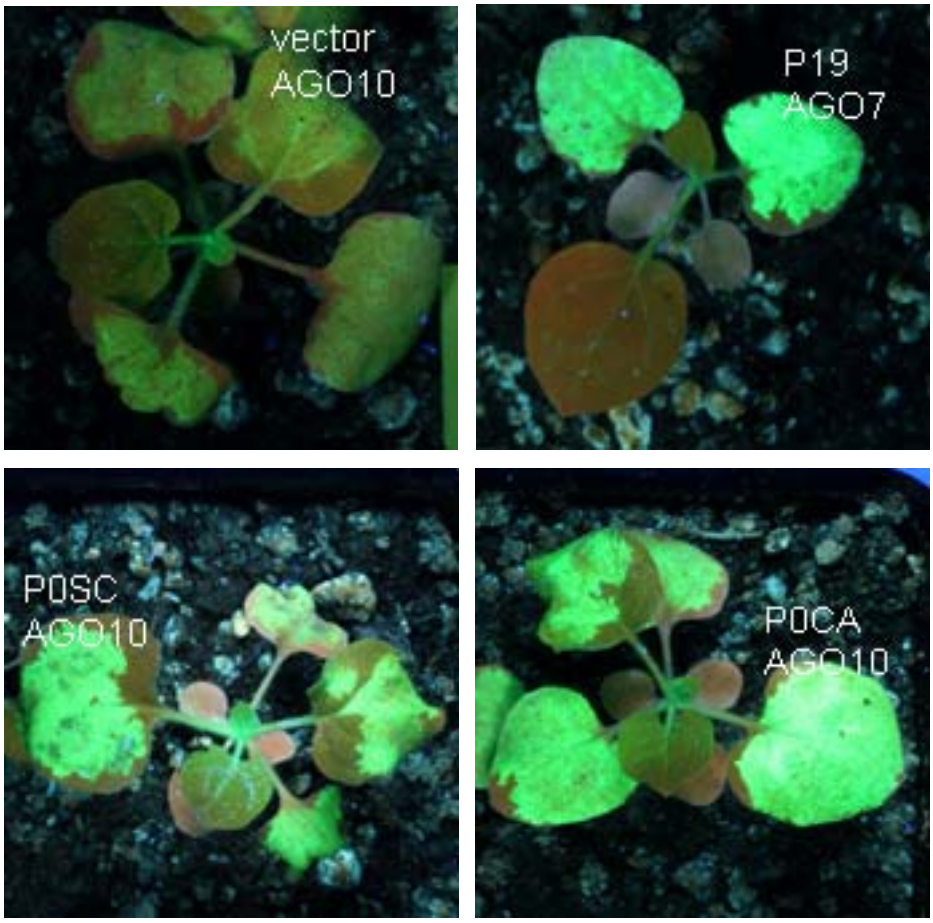
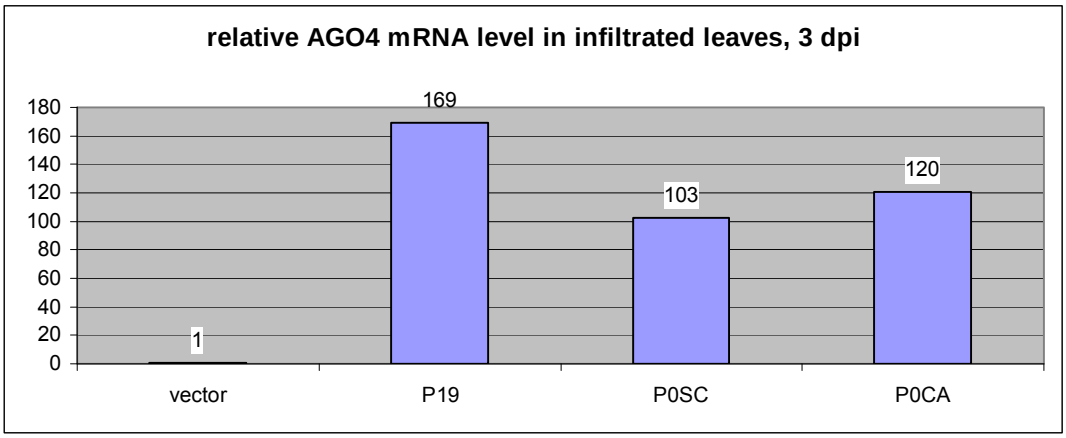


Figure 10: *Agrobacterium* leaf infiltration in 16c *N. benthamiana*
sGFP, sense GFP; vector, empty vector negative control; p19,
Tomato bushy stunt virus p19 protein; AGO4/7/10, *A. thaliana* Argonaute 4/7/10

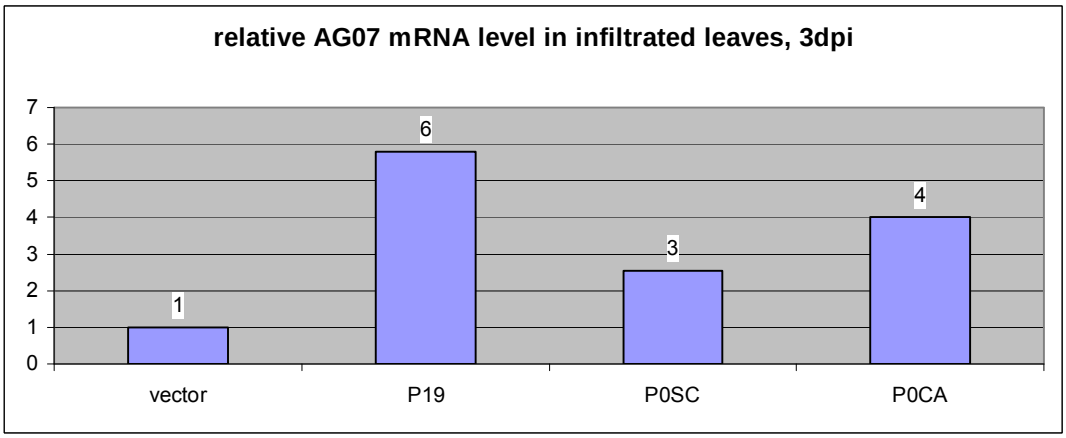
3.3. mRNA analysis

Total RNA was extracted 3 days post infiltration from infiltrated 16c *N. benthamiana* leaves, Argonaute mRNA levels were analyzed by qRT-PCR and normalized to actin. This approach was chosen to secure transient coexpression of the epitope-tagged Argonaute constructs in the presence of the viral silencing suppressors. In comparison with both P0 proteins, AGO mRNA levels were increased in the presence of p19.

A.



B.



C.

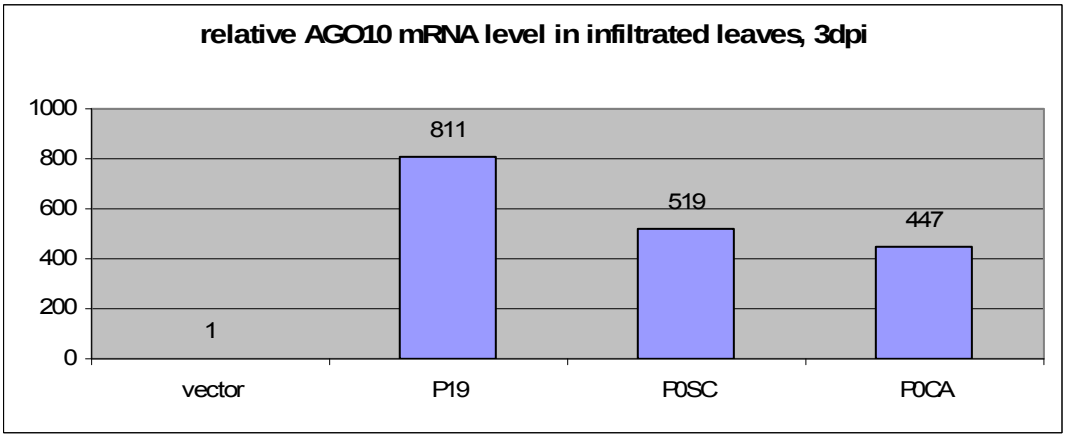


Figure 11: Argonaute mRNA levels in infiltrated leaves 3 dpi. AGO mRNA levels determined by qRT-PCr, normalized to actin. vector, empty vector negative control; P19, p19 positive control,

3.4. Protein extraction and Western Blot analysis

To investigate the proposed ability of $P0^{SC}$ to destabilize *Arabidopsis* Argonautes, the cDNAs of AGO4, AGO7, and AGO10 were transiently coexpressed with $P0^{SC}$ and further silencing suppressors in *N. benthamiana* leaves. The level of each epitope tagged AGO protein in crude protein extracts was tested with anti-FLAG antibodies.

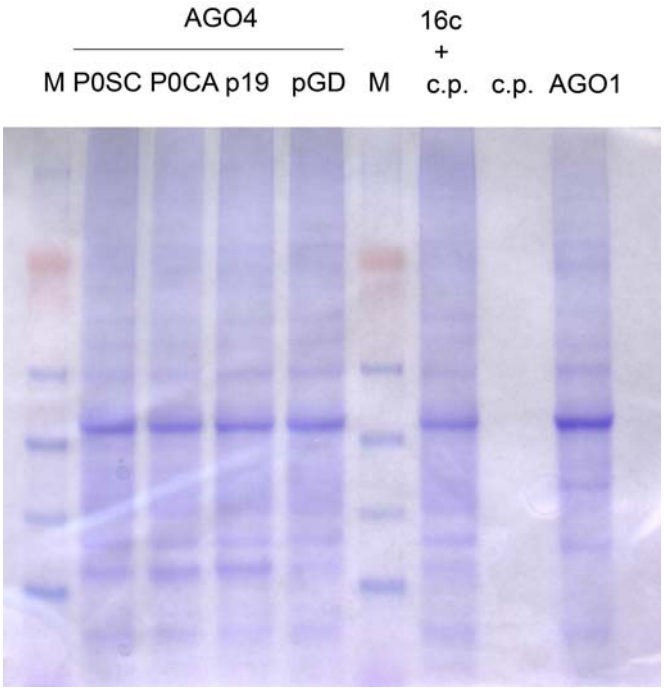
The protein extraction protocol was modified several times, because extractions conducted with Buffer I had only a very low protein concentration of 0.35 – 0.65 µg/µl, which did not enable us to use the maximum protein amount in the following Western Blots. Slight modifications like reduction of the buffer volume did not improve the protein content of the samples. Therefore, we decided to use Buffer II, which was previously described by Bortolamiol et al (2007), to achieve this improvement. This modification in the protocol resulted in a drastic change in the protein content of the crude extracts.

Although, the protein content of the crude extracts was clearly sufficient, we were not able to detect specific signals in subsequent Western blots. Since Dot blots with N-terminal Met-FLAG-BAP control protein demonstrated functional integrity of the used antibodies, several modifications were introduced to overcome these difficulties.

One approach was to increase the protein amount per lane and consequently the electrophoresis time, in case sufficient target protein was not remaining in the protein extracts. Moreover, an additional denaturation step was introduced to the sample preparation protocol to guarantee accessibility to the FLAG sequence of the modified Argonaute proteins. These changes did not improve detection of the FLAG epitope on Western blots.

In the course of the experiments, loss of target protein became evident; this prompted us to set up additional controls to test this possibility. The first control was to add N-terminal Met-FLAG-BAP control protein to non infiltrated *N. benthamiana* 16c tissue and conduct protein extraction as described for the other samples. By

A.



B.

.

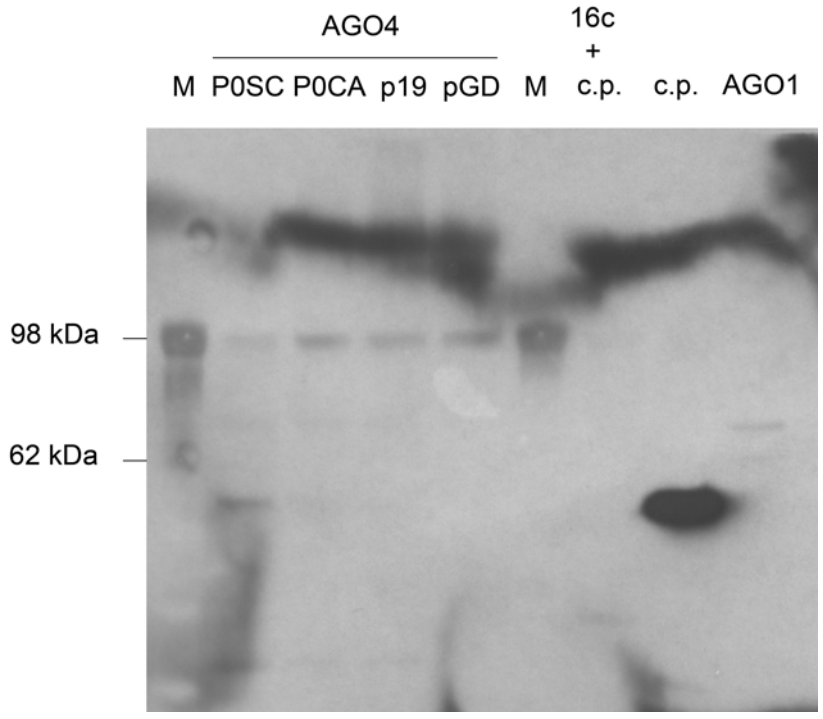


Figure 12: Sensitivity of *Arabidopsis* Argonaute 4 to P0-mediated degradation. Different FLAG-Argonaute constructs were expressed transiently with several silencing suppressors in *N. benthamiana* leaves. Total protein was extracted 3 dpi and level of Argonaute proteins was tested by western blots with ANTI-FLAG M2 antibody. A. Equal loading was controlled by coomassie brilliant blue staining of a replica gel. B. Immunodetection. P0SC, *sugarcane yellow leaf virus* $P0^{SC}$; P0CA, *cucurbit aphid-borne yellow virus* $P0^{CA}$; p19, *Tomato bushy stunt virus*p19 protein; pGD, empty vector negative control; AGO1/4, *A. thaliana* Argonaute 1/4; c.p., N-terminal Met-FLAG-BAP control protein; 16c + c.p., non- infiltrated N. benthamiana tissue + control protein

contrast, control protein alone was observed on a separate lane. Additionally, protein extracts from three week old *Arabidopsis* plants containing an FLAG AGO1 construct, which were a generous gift of Dr. Baulcombe, were analyzed, because these plants were objects of several studies and would serve as an additional indicator of target protein loss.

All samples from coinfiltrations with tagged Argonautes and tested suppressors, showed several non specific bands with signals around 100 kDa being the most prominent one. In FLAG AGO1 samples no signals were detected around the expected 116 kDa. The comparison of control protein alone and control protein in *N. benthamiana* 16c extract revealed an enormous protein loss in *N. benthamiana* 16c, because only immunodetection of control protein alone gave strong signals.

4. Discussion

During the last decade great efforts were undertaken to understand how viruses are able to overcome plants' adaptive immune system also referred to as posttranscriptional gene silencing. Viral silencing suppressor proteins act at distinct steps of the PTGS pathway and it has become apparent that Argonaute 1 protein could serve as a target in silencing suppression strategies. Zhang et al (2006) reported that *cucumber mosaic virus*-encoded protein 2b directly interacts with AGO1 and inhibits its slicer activity in RISC to circumvent host defense.

In contrast to this inhibitory mechanism, members of the P0 protein family were shown to use proteolysis as a strategy to suppress posttranscriptional gene silencing (Bortolamiol et al. 2007). Although these proteins do not share many similarities they are characterized by an F-box-like domain, whose functional integrity is necessary to mediate viral pathogenicity. The proposed F-box-like domain is LPXXI/LX_nP and it was shown that P0 proteins interact via this domain with *Arabidopsis* ASK1 and ASK2, which are core components of the SCF ubiquitin ligase complex (Pazhouhandeh et al. 2006). The finding of P0 being the mediator for AGO1 degradation, supported the theory that P0 adapts the SCF ubiqitin ligase complex to overcome post transcriptional gene silencing by attacking one of its core components (Bortolamiol et al. 2007). This straightforward model of a hostile takeover of the host proteasome-dependent degradation pathway is now challenged, since it has been shown that AGO1 degradation is proteasome independent (Baumberger et al. 2007).

$P0^{SC}$, like the previously characterized P0 proteins, contains a possible F-box-like domain and, in addition to suppression of local silencing, $P0^{SC}$ exhibits novel activities including suppression of systemic silencing and induction of cell death in infiltrated areas (Mangwende et al. unpublished data).

Considering that proteins interacting with $P0^{SC}$ could not be identified in previous experiments, this raised the question whether we could apply the concept of P0 mediated Argonaute degradation to $P0^{SC}$ or not.

4.1. *A. thaliana* experiments

A. thaliana in planta coexpression experiments served as initial framework for our experiments. The availability of a transgenic Arabidopsis line expressing an epitope tagged variant of one target gene, FLAG AGO1, encouraged us to design two constructs containing $P0^{SC}$ under the control of a constitutive and an inducible promoter, which were used to transform *Arabidopsis* wild type plants. If $P0^{SC}$ mediates AGO1 degradation, it would be possible to detect a reduction of the target via western blots.

We were not able to obtain any transgenic lines constitutively expressing $P0^{SC}$, which seems to be consistent with the previously described cell death mediating trait of $P0^{SC}$. Although this conclusion cannot be proven, it is supported by experiments, where transformed seedlings constitutively expressing $P0^{BW}$ - a member of the P0 protein family without cell death mediating capabilities – showed strong developmental defects and were not able to reach juvenile stages (Bortolamiol et al. 2007). In the course of the project planning stage, it became clear that this outcome was very likely to happen and to overcome this difficulty, the need of an inducible expression method was considered critical. This approach would enable us to produce plants harboring $P0^{SC}$ while avoiding possible deleterious effects of constitutive expression. For this reason, plants were transformed with a heat shock inducible construct and two hygromycin resistant transgenic lines were isolated. Due to a lack of time, measurement of transgene expression by qRT-PCR could not be conducted and needs to be addressed in the future. If these transformants express $P0^{SC}$ at sufficient levels when induced, they could be crossed with the provided FLAG AGO1 expressing line and coexpression experiments could be conducted.

Although it seemed to be an effective approach to test our assumptions, the chosen heat shock inducible system might cause complications, because the physical stress of heat shock induction probably induces the expression of several other genes thereby interfering with the process under investigation (Curtis and Grossniklaus. 2003). Therefore, a FLAG-AGO1 vector was constructed similar to the three other Argonaute vectors constructed for Arabidopsis transformation. All four FLAG-AGO

vectors were used in *N. benthamiana* leaf infiltration experiments. This procedure allowed us to avoid both long-term constitutive expression of the possibly cytotoxic $P0^{SC}$ protein and heat shock induced perturbations of gene expression. Furthermore, it offered the possibility of more rapid experimental results, as compared to making stable transgenic Arabidopsis, confirming transgene integration and expression, followed by sexual crosses required to get functional copies of both transgenes into individual test plants.

4.2. *N. benthamiana* leaf infiltrations

Since it became apparent that the *Arabidopsis* experiments would not provide required results to evaluate the hypothesis of $P0^{SC}$ mediated Argonaute degradation in the time available, *N. benthamiana* was chosen as model system. Transient coexpression of silencing suppressors and tagged target proteins proved to be a suitable and timesaving tool to investigate this ability of $P0^{BW}$ and $P0^{CA}$ (Baumberger et al. 2007, Bortolamiol et al.2007).

For this purpose, an experimental setup had to be created, which ensured the simultaneous co-expression of a silencing suppressor and one of the chosen Argonaute proteins and allowed controls at different stages. Since there was no a $P0^{SC}$ specific antibody available at the time these experiments were undertaken, the functional integrity of each suppressor was verified by their ability to suppress silencing of a sGFP construct. In contrast to the negative control, each suppressor exhibited this capability and consequently, it was assumed that each silencing suppressor was active. The presence of each tagged Argonaute transcript was confirmed by qRT-PCR, which assured that both components of the experiment were present. Thereupon, total protein from infiltrated N. benthamiana leaves was extracted and the level of each tagged Argonaute protein was tested by anti FLAG antibodies to investigate the proposed ability of $P0^{SC}$ to target Argonaute proteins for degradation. If $P0^{SC}$ possesses this ability, it should be possible to detect reduced accumulation or complete degradation of the target in Western Blots. Although

targeted decay of AGO1 *in planta* has been proven only for $P0^{BW}$, it is tempting to assume a similar behavior in $P0^{CA}$, because GST-tagged $P0^{CA}$ co-precipitates with FLAG-AGO1 in pull down assays (Bortolamniol et al 2007). Moreover, Baumberger et al. (2007) reported that $P0^{BW}$ mediates degradation of AGO2, AGO4-6, and AGO9 in addition to AGO1. As $P0^{SC}$ suppressor activities as determined from *N. benthamiana* leaf infiltration experiments differ from those of $P0^{BW}$ and $P0^{CA}$, it was unknown whether interactions with Argonaute proteins would be conserved in $P0^{SC}$.

Although, several extraction methods were applied, it became apparent that the epitope tagged target proteins were lost during the extraction, because we were not able to obtain specific signals in subsequent Western blots. The most prominent signals in all combinations of a test suppressor with an Argonaute were detected at ~100kDa, which matches approximately the atomic mass of the target.

At first glance, this observation might mislead to the assumption that $P0^{SC}$ as well as $P0^{CA}$ are not able to mediate degradation of Argonaute proteins, but Baumberger et al. (2007) mention that some proteins from the crude extracts of infiltrated *N. benthamiana* leaves possess similar electrophoretic mobility and are reacting with the anti-FLAG antibody, thereby overlaying the signals of the tagged Argonaute proteins. Therefore, immunopurifications from the crude extracts should be conducted in future reruns of this experiment to avoid loss of target protein and prevent crossreacting nonspecific bands.

In case, future experiments would confirm that $P0^{SC}$ is able to mediate Argonaute degradation, it will be interesting to evaluate, if the presence of a PAZ and ND domain in AGO is essential for this ability like it has been shown for $P0^{BW}$ (Baumberger et al. 2007). Mangwende et al. (unpublished data) suggested that $P0^{SC}$ might inhibit one or more Dicer enzymes, because it suppresses the accumulation of all size classes of siRNA. Since, Dicer enzymes contain a PAZ domain (Yan et al. 2003), it might be worth investigating whether this domain by itself is sufficient to be targeted for degradation, contradicting inhibition of Dicer by $P0^{SC}$, which could explain the unique cell death induction by $P0^{SC}$. Consequently, future projects could cover this interesting aspect of silencing suppression.

A less speculative approach is suggested by the newly available $P0^{SC}$ specific antibody. Proteins interacting with $P0^{SC}$ could be isolated by coimmunoprecipitation and analyzed by mass spectroscopy.

Although we were not able to verify the proposed concept of $P0^{SC}$ mediated degradation of Argonaute proteins, we framed the fundamental principles for future experiments by pointing out the main obstacles. Nevertheless, it will remain challenging to investigate how this potent silencing suppressor enables *Sugarcane yellow leaf virus* to evade posttranscriptional gene silencing.

5. References

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Appendix

6. Zusammenfassung

Unter den Mitgliedern der Luteoviridae hat sich das P0 protein als ausgesprochen heterolog herausgestellt. Bei drei getesteten Mitgliedern dieser Familie (*Potato leaf roll virus*, PLRV; *Beet western yellows virus*, BWYV; *Cucurbit aphid-borne yellows virus*, CABYV) wurde bewiesen, dass das P0 Protein für die Überwindung von local posttranscriptional gene silencing (PTGS) verantwortlich ist.

Obwohl diese Proteine nur wenige Gemeinsamkeiten aufweisen, besitzen sie alle eine F-box-like domain, deren funktionale Integrität notwendig ist zur Vermittelung viraler Pathogenität. Um dieses Ziel zu erreichen, wird die Strategie verfolgt Arabidopsis Argonaute 1 zur Zerstörung durch Proteolyse bereitzustellen.

P0 SCYLV besitzt ähnlich den anderen beschriebenen P0 Proteinen ebenfalls eine F-box-like domain und zeigt - neben der Fähigkeit zur Überwindung von local silencing – neuartige Eigenschaften wie Fähigkeit zur Überwindung von systemic silencing und Zelltodinduktion in den infiltrierten Bereichen.

In Anbetracht der Tatsache, dass bis jetzt noch keine Interaktionspartner von P0 SCYLV identifiziert werden konnten, wollten wir im Rahmen dieser Arbeit die Frage verfolgen, ob das Konzept eines durch P0 vermittelten Argonaute Abbaus auch auf P0 SCYLV übertragbar ist.

7. Abstract

The P0 protein is highly heterologous among different members of the Luteoviridae. However, in three tested members of this family (*Potato leaf roll virus*, PLRV; *Beet western yellows virus*, BWYV; and *Cucurbit aphid-borne yellows virus*, CABYV) the P0 protein suppresses local posttranscriptional gene silencing (PTGS).

Although these proteins do not share many similarities they are characterized by an F-box-like domain, whose functional integrity is necessary to mediate viral pathogenicity; and these proteins target *Arabidopsis* Argonaute 1 for proteolysis.

P0 SCYLV, like the previously characterized P0 proteins, contains an F-box-like domain and, in addition to suppression of local silencing, $P0^{SC}$ exhibits novel activities including suppression of systemic silencing and induction of cell death in infiltrated areas.

Considering that proteins interacting with P0 SCYLV could not be identified in previous experiments, this raised the question whether we could apply the concept of P0 mediated Argonaute degradation to P0 SCYLV or not.

8. Resume

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