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Co-introduction of an antisense gene for an endogenous seed storage protein can increase expression of a transgene in *Arabidopsis thaliana* seeds

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Abstract We have investigated whether the expression in *Arabidopsis thaliana* seeds of a transgene (the *Phaseolus vulgaris arcelin (arc)5-I* gene) could be enhanced by the simultaneous introduction of an antisense gene for an endogenous seed storage protein (2S albumin). Seeds of plants transformed with both the *arc5-I* gene and a 2S albumin antisense gene contained reduced amounts of 2S albumins and increased arcelin-5 (Arc5) accumulation levels compared to lines harboring the *arc5-I* gene only. Arc5 production could be enhanced to more than 24% of the total seed protein content, suggesting that antisense technology could be of great utility to favor high expression of transgenes.

Key words: Antisense repression; Arcelin; Seed protein; Transgene expression level; *Arabidopsis thaliana*; *Phaseolus vulgaris*

1. Introduction

A variety of seed storage protein genes has already been expressed in heterologous plant hosts. In general, the expression of the introduced genes is temporally and spatially regulated in a normal way and the corresponding protein products are correctly processed in the developing seeds. In contrast, the amount of accumulated proteins is generally low: usually not more than a few percent of total seed protein [1]. To improve the nutritional quality of seeds or to use seeds as a production system for pharmaceutically or industrially interesting products, higher transgene expression levels are generally required. Increased synthesis and accumulation of transgene-derived seed proteins could be obtained by increasing the transgene copy number or by using a more active promoter to drive transgene expression. Alternatively, a gene that encodes a major endogenous seed storage protein could be repressed or inactivated, thereby elevating the levels of other endogenous or transgene-derived seed proteins. One way to obtain this result is through antisense technology, which is used extensively in plants to investigate the biological functions of the corresponding sense genes or to improve economically important characteristics [2]. Recently, the usefulness of this approach for modifying the quantity of storage components was demonstrated in *Oryza sativa* [3] and *Brassica napus* [4,5]. In the latter species, seeds of plants transformed with an antisense gene against one of the two major storage proteins (napins [4] and cruciferins [5]) often contained substantially to completely repressed levels of the targeted protein and concomitantly, an increase in the content of the other seed storage protein fraction. Total protein and lipid contents of transgenic seeds did not differ significantly from that of normal seeds.

The crucifer *Arabidopsis thaliana* also contains two major classes of seed storage proteins, the 2S albumins and the 12S cruciferins [6] that are similar in size and subunit composition to the napins and cruciferins of *B. napus*. The major storage proteins in *A. thaliana* seeds are the 2S albumins, which are encoded by a small multigene family [7]. *A. thaliana* has already been used as a heterologous expression system for diverse seed proteins [8–11]. Recently, we have introduced the arcelin-5(*arc5-I*) gene into *A. thaliana* to assess the utility of arcelin-5 (Arc5) signals to obtain high seed-specific expression in transgenic plants [12]. Arc5 is an abundant seed storage protein found in a few wild common bean (*Phaseolus vulgaris*) genotypes [13] and is encoded by two very similar genes: the *arc5-I* gene that encodes the Arc5a protein and the *arc5-II* gene that encodes Arc5b together with a minor non-glycosylated isoform, Arc5c [14]. The Arc5 protein fraction represents 30–40% of the total protein content in seeds of these wild genotypes. Also in seeds of transgenic *A. thaliana*, high accumulation levels of the Arc5 protein (up to 15% of the total seed protein) were observed [12]. However, the protein/mRNA ratio for the *arc5-I* gene was substantially lower than for the endogenous 2S albumin genes. An analogous situation was reported for a chimeric Brazil nut 2S albumin transgene when expressed in *A. thaliana* seeds [10]. This could be caused by a difference in protein stability or by a reduced competitiveness of the transgene-derived products for the (post-)translational machinery of *A. thaliana*. Therefore, we investigated whether introducing an antisense gene for a major endogenous seed storage protein would substantially enhance transgene product accumulation or, on the contrary, would mainly favor expression of other endogenous seed-specific genes.

2. Materials and methods

2.1. Transformation of A. thaliana with arc5 genes

In planta, transformation of the *A. thaliana* genotype Columbia-O

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Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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[15] was carried out with the *Agrobacterium tumefaciens* strain C58C1Rif containing the helper plasmid pMP90 [16] and harboring either the binary plasmid pATARC4-A, pATARC4-SAA or pATARC4-SAS. These plasmids are all derived from the pATAG4 plasmid [12]. pATARC4-A (Fig. 1) contains the neomycin phosphotransferase II gene between the T-DNA borders (*nptII*), under control of the nopaline synthase promoter and the octopine synthase 3′ termination and polyadenylation signals, and a genomic *Acc*I fragment of the *arc5-I* gene comprising the *arc5-I* coding region flanked by 1.8 and 1.5 kb of 5′ and 3′ regulatory sequences, respectively [17]. The two other plasmids contain in addition an antisense gene for the *A. thaliana* 2S2 albumin (*at2S2*) [7]. In the SAA construct, the *at2S2* coding sequence was transcriptionally fused, in antisense, to the 5′ and 3′ flanking sequences of *arc5-I*, whereas in the SAS construct, it was placed in antisense between its own 5′ and 3′ flanking sequences (Fig. 1). Transgenic seedlings (T_1 generation) were selected on growth medium [18] containing 50 μg/ml kanamycin (Sigma, St. Louis, MO, USA) and 200 μg/ml cefotaxime (Claforan, Hoechst AG, Frankfurt am Main, Germany). The T_2 segregation was analyzed under the same conditions. The number of integrated T-DNAs was determined by DNA gel blot analysis on total DNA of T_2 seedlings (prepared as described in [19]) with use of the Gene Images kit (Amersham Life Science, Aylesbury, UK).

2.2. Quantification of Arc5a levels in transgenic A. thaliana seeds

Crude seed protein extracts were obtained as previously described [12]. In plant organs of which the bulk of total protein is made up of only a few different proteins, as is the case for *A. thaliana* seeds, different methods to estimate the total protein content can give widely varying results. Quantification of a specific protein as percentage of the total extractable protein content then becomes problematic (Table 1). Therefore, we designed a protocol that should give reliable data (Table 1, method 1). A large-scale protein extraction was performed on seeds of *A. thaliana* plants. Salts (from the extraction buffer) and low-molecular weight seed compounds were removed by gel filtration (NAP-10, Pharmacia Biotech, Uppsala, Sweden) and extracts were subsequently lyophilized. Total extracted protein was weighed and absolute Arc5a amounts were determined by densitometry scanning of Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [20]. Arc5 proteins purified from the wild *P. vulgaris* genotype were used to construct a standard curve. As large amounts of seeds are required for this method, only one transgenic line for each type of construct was analyzed in this way. Data were subsequently compared with data obtained by more convenient quantification methods (Table 1). It was found that reliable Arc5a quantification data for all lines could be obtained by using a high performance liquid chromatography (HPLC)-based method (Table 1, method 2). To estimate Arc5a levels in A and SAA lines, also a Lowry/Western-based method gave valid results (Table 1, method 3). As the latter method is less time and material consuming, it was adopted to analyze A and SAA lines. Both techniques are independent of the buffer system used to extract seed proteins (data not shown). Below, both protocols are described in detail. Each transgenic line was examined at least two times for Arc5a levels (approximately 500 seeds per assay).

Crude seed extracts of SAS lines (acidified with 0.1% trifluoroacetic acid) were analyzed via reversed-phase chromatography on a PLRP-S column (150×4.6 mm, 8 μm particles, 300 Å pore size: Polymer Laboratories, Church Stretton, UK) connected to a Vista 5500 HPLC system (Varian, San Fernando, CA, USA). A gradient from 0 to 70% acetonitrile (in 0.1% trifluoroacetic acid) was used to separate the extracts. The presence of proteins (and other compounds) was monitored by absorbance at 215 nm using a Varian 9060 Polychrom diode array detector and recorded (Fig. 2B and C). Peaks that corresponded to protein products were identified through SDS-PAGE (Fig. 2A). Peak areas were calculated using a Varian 4290 integrator. In this way, Arc5a accumulation levels (as % of total extractable seed protein) could be estimated.

For A and SAA lines, the total protein quantity in the crude extracts was determined by the Lowry method using the DC Protein Assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. Expression levels of Arc5 proteins were estimated (as percentage of total extractable seed protein) by Western blot analysis [21] using a rabbit polyclonal anti-Arc5 antiserum. Arc5 proteins purified from wild *P. vulgaris* were used to construct the standard curve.

2.3. Detection and quantification of arc5-I and at2S mRNA in transgenic A. thaliana siliques

Siliques at stages D, E and DS (as designated in [22]) were harvested and pooled. Total RNA was prepared as described in [23]. mRNA steady state levels were determined by slot blot analysis following the method described in [22], except that a non-radioactive detection method was used (Gene Images, Amersham). Levels of both *arc5-I* and the endogenous 2S albumin transcripts were estimated with an Arc5 RNA probe (corresponding to an internal cDNA fragment: [14]) or a 2S2 RNA probe (covering the complete coding sequence of the *A. thaliana at2S2* gene: [7]), respectively. Fluorescein-labelled (Roche Diagnostics, Brussels, Belgium) antisense RNA was used as a probe and was synthesized with the Riboprobe combination system SP6/T7 (Promega, Madison, WI, USA). After hybridization and detection, each signal on the film was quantified by densitometry scanning with the Imagemaster VDS software (Pharmacia). Each RNA preparation was examined at least three times for *arc5-I* or *at2S* steady state levels. Because the *at2S2* probe is probably not specific to the *at2S2* transcripts alone and might also hybridize with transcripts from the other endogenous 2S albumin genes [22], we used the term *at2S* transcripts rather than *at2S2* transcripts.

3. Results

A. thaliana plants were transformed with the following constructs: (i) the A construct, which harbors the *arc5-I* gene, (ii) the SAA construct, which contains, in addition to the *arc5-I* gene, a 2S2 albumin antisense gene under control of the 5′ and 3′ regulatory sequences of the *arc5-I* gene and (iii) the SAS construct, which contains the *arc5-I* gene and a 2S2 albumin antisense gene under control of its own regulatory sequences (Fig. 1). Transgenic lines were selected for the presence of one transgenic locus with intact T-DNAs. There-

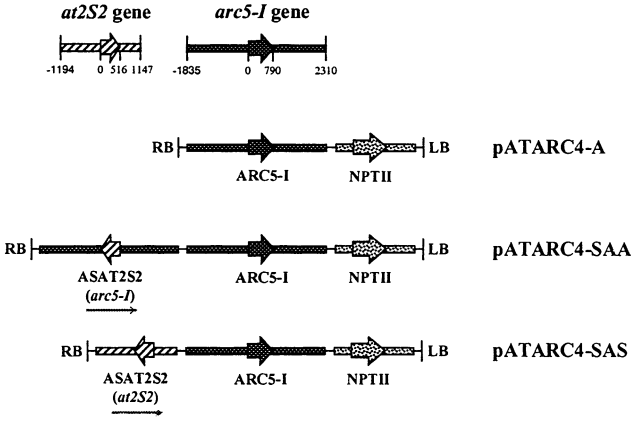

Fig. 1. Schematic representation of the T-DNA of the binary vectors pATARC4-A, pATARC4-SAA and pATARC4-SAS. Coding regions are indicated by arrows and 5′ and 3′ flanking regulatory sequences as blocks. Numbers correspond with positions in the *arc5-I* or *at2S2* gene relative to their respective translation start sites. All T-DNA constructs contain the *pnos-nptII-3′ocs* chimeric gene (NPTII) and the *arc5-I* gene (ARC5-I). pATARC4-SAA and pATARC4-SAS contain in addition an antisense gene for the 2S2 albumin (ASAT2S2). The origin of the regulatory sequences of the antisense gene is indicated between brackets and a gray arrow gives the transcriptional direction. In pATARC4-SAA, the *at2S2* coding sequence (from position −30 to 541) was transcriptionally fused, in antisense, to the *arc5-I* 5′ and 3′ flanking sequences (from positions −1835 to −5 and from 685 to 2010, respectively). In pATARC4-SAS, the *at2S2* coding region (from position −30 to 541) was placed in antisense between its own 5′ and 3′ flanking sequences (from positions −1194 to 15 and from 516 to 1147, respectively). LB and RB, left and right border repeat of the T-DNA.

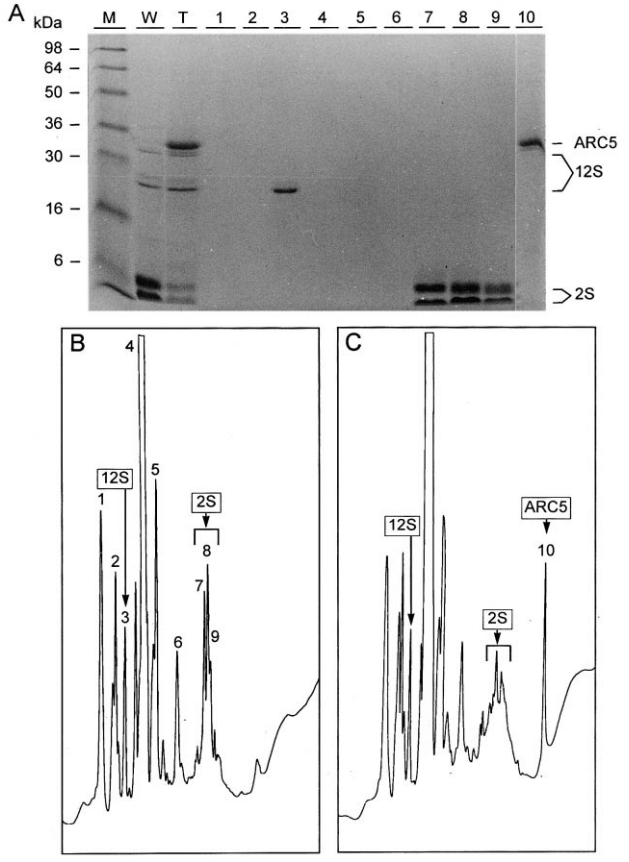

Fig. 2. HPLC analysis of crude *A. thaliana* seed protein extracts. (A) Coomassie blue-stained SDS-PAGE analysis of crude seed protein extracts of the non-transformed Columbia-O genotype (lane W) and of the transgenic line SAS-203 (lane T). Lanes 1–10 correspond to peaks 1–9 in B and peak 10 in C, lane M contains marker proteins (molecular mass indicated on the left in kDa). (B) HPLC analysis of a crude seed protein extract of the non-transformed Columbia-O genotype. (C) HPLC analysis of a crude seed protein extract of the transgenic line SAS-203. 2S, 2S albumins; 12S, 12S cruciferins; ARC5, Arc5a.

after, seeds and developing siliques of homozygous transgenic plants were assessed for their storage protein and storage protein mRNA contents. Results are summarized in Table 2.

SDS-PAGE indicated that lines transformed with the SAS construct showed a reduction in relative amounts of the endogenous 2S albumins and an increase in relative amounts of the Arc5a protein (Fig. 3A). Indeed, by using HPLC-based quantification assays, SAS lines were shown to possess Arc5a levels ranging from 7.8 to 24.6% of total seed protein (Table 2), which is a clear enhancement (Fig. 4) compared to lines harboring the A construct (Table 2) and lines transformed with other genomic fragments of the *arc5-I* gene (i.e. BM and E constructs [12] that are identical to the A construct except that they contain a shorter and a longer fragment of the *arc5-I* genomic clone [17], respectively). The BM, A and E lines all accumulated Arc5a in the range of 1–15% of total seed protein. The effect of the antisense gene on expression of the 2S albumin genes was even more pronounced on the mRNA level. *Arc5-I*/*at2S* mRNA ratios were much higher in SAS lines than in A lines (Table 2) or BM and E lines [12]. This result was primarily caused by a decrease in 2S albumin transcripts as could be deduced from absolute steady state levels (data not shown).

In contrast, SDS-PAGE indicated that in transgenic lines containing the SAA construct, the amount of 2S albumins was not or hardly reduced (Fig. 3B). Moreover, SAA lines had only low amounts of Arc5a (except line SAA-242), with levels ranging from 0.1 to 3.2% of total seed protein (Table 2). This amount was lower than in the A, BM and E lines, of which a majority produced Arc to levels of 5% or more (Fig. 4). RNA slot blot analysis showed that this was related to low steady state levels of *arc5-I* transcripts. SAA lines with low Arc5a levels had a low *arc5-I*/*at2S* mRNA ratio (Table 2).

Table 1
Comparison of different techniques to quantify Arc5a protein in *A. thaliana* seeds as percentage of the total extractable protein

Quantification	Arc5a accumulation levels (% total seed protein)		
	A-312	SAA-221	SAS-208
Method 1[a]	13.9	2.6	22.2
Method 2[a]	13.7	4.5	22.9
Method 3[a]	12.2	2.2	9.6
Method 4[b]	22.6	14.7	45.8

[a]See Section 2.
[b]Relative percentage of Arc5a protein levels in a crude seed extract as estimated by densitometry scanning of Coomassie blue-stained SDS-PAGE.

4. Discussion

Previous reports have already shown the feasibility of in-

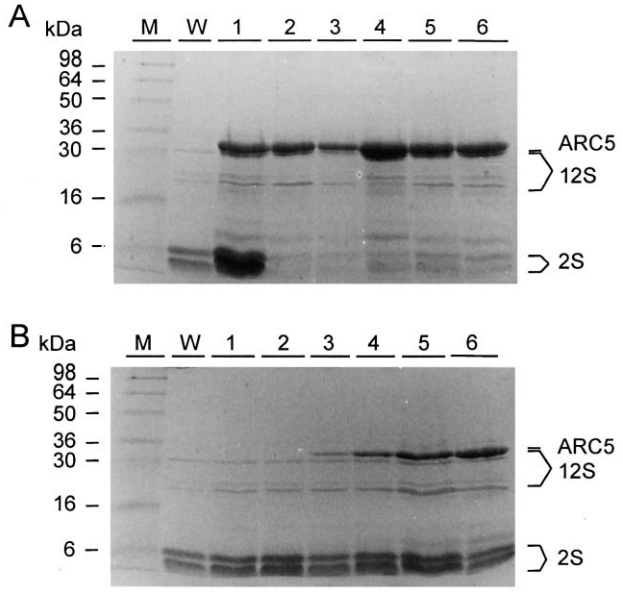

Fig. 3. SDS-PAGE analysis of crude *A. thaliana* seed protein extracts. Proteins are visualized by Coomassie blue staining. Lanes shown in A contain crude protein extracts of seeds of the non-transformed Columbia-O genotype (lane W) and of transgenic lines harboring the A (A-312, lane 1) or SAS construct (SAS-102, SAS-203, SAS-208, SAS-209 and SAS-211, lanes 2–6, respectively). Lane M contains marker proteins (molecular mass indicated on the left in kDa). Lanes shown in B contain crude protein extracts of seeds of non-transformed (lane W) and transgenic lines harboring the SAA construct (SAA-239, SAA-231, SAA-217, SAA-221, SAA-219 and SAA-242, lanes 1–6, respectively). Lane M contains marker proteins (molecular mass indicated on the left in kDa). 2S, 2S albumins; 12S, 12S cruciferins; ARC5, Arc5a.

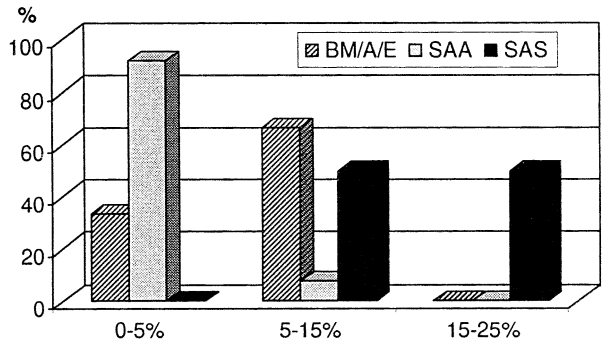

Fig. 4. The effect of the 2S2 albumin antisense genes on Arc5a accumulation. For each type of construct (BM/A/E, SAA and SAS), the percentages of the transgenic lines (ordinate) accumulating Arc5a to levels of 0–5%, 5–15% or 15–25% of total seed protein (abscissa) are presented. Data of transgenic lines harboring the BM, A and E constructs were pooled.

creasing accumulation levels of certain endogenous seed storage proteins by reducing the production of another seed protein by an antisense gene [3–5]. Here, we addressed the question whether the biosynthetic capacity that becomes available in seeds as a result of repressing a major endogenous protein would be allocated to the synthesis of a transgene-encoded protein. Therefore, we transformed *A. thaliana* with the *arc5-I* gene and an antisense gene for the 2S2 albumin, both located on the same T-DNA. Of the known 2S albumin genes [7], the *at2S2* gene is the best target for antisense repression as it is expressed throughout the embryo and contributes to the bulk of the 2S mRNA (40–55%) [22].

In SAS lines, which harbor the antisense gene under control of the *at2S2* regulatory sequences, an important, but not complete, reduction in 2S albumins (both on the protein and the mRNA level) can be observed, which demonstrates the functionality of the antisense gene. Whether the repression was restricted to 2S2 albumins or whether other 2S isoforms were also affected was not further investigated. The fact that only partial repression was obtained might be attributed to the sequence diversity between the different 2S isoforms [7], although it is also possible that expression levels of the antisense gene were insufficient to inactivate all 2S albumin synthesis. With respect to the transgene expression levels, SAS lines were clearly shown to contain elevated accumulation levels of the Arc5a protein compared to lines harboring only the *arc5-I* gene. Arc5a production could be enhanced to more than 24% of total seed protein, showing that the reduction in 2S albumin levels is, at least partially, compensated by an increase in the accumulation of the transgene-derived product.

The endogenous *at2S2* promoter could reduce 2S albumin expression significantly when placed in front of the 2S2 antisense gene. In contrast, the *arc5-I* regulatory sequences did not reduce 2S albumin expression when flanking the same antisense gene (SAA lines). This observation does not imply that the *arc5-I* flanking sequences per se would not be suitable to obtain repression of the endogenous seed protein genes. The fact that in general no effect on 2S albumin accumulation is observed could be related to the tandem repeat organization of the chimeric antisense gene and the *arc5-I* gene. Indeed, the presence of this chimeric antisense gene seems to cause lower Arc5a accumulation levels in SAA lines compared to lines expressing the *arc5-I* gene only, suggesting that some kind of homology-dependent gene silencing event occurs.

Table 2
Characterization of transgenic *A. thaliana* plants harboring one transgenic locus

Line	Number of T-DNA copies[a]	Arc5a protein level	mRNA ratio *arc5-I*/*at2S*
A-312	1	12.2 ± 1.3	0.8
A-TR108	2 (IRR)	1.4 ± 0.9	ND
A-302	≥ 3	1.7 ± 1.0	0.1
SAA-231	1	0.1 ± 0.0	ND
SAA-226	1	1.1 ± 1.3	ND
SAA-249	1	1.2 ± 0.5	ND
SAA-234	1	1.5[b]	ND
SAA-202	1	1.7 ± 0.5	ND
SAA-222	1	2.9[b]	ND
SAA-219	1	3.2 ± 0.8	2.7
SAA-242	1	14.5 ± 1.1	ND
SAA-239	2 (TR)	0.1 ± 0.0	ND
SAA-217	2 (TR)	1.3 ± 0.4	0.2
SAA-241	2 (IRR)	2.1[b]	ND
SAA-230	≥ 3	0.3 ± 0.1	0.2
SAA-221	≥ 3	2.2 ± 0.8	ND
SAS-107	1	7.8[b]	4.9
SAS-212	1	15.9 ± 0.8	11.8
SAS-105	2 (IRL)	11.6 ± 5.0	ND
SAS-102	2 (TR)	12.7 ± 1.9	ND
SAS-208	≥ 3	22.9[b]	ND
SAS-209	≥ 3	12.5 ± 2.9	ND
SAS-203	≥ 3	24.6 ± 0.0	ND
SAS-211	≥ 3	16.7 ± 1.3	38.5

Transgenic lines marked with A, SAA and SAS contain T-DNA inserts from pATARC4-A, pATARC4-SAA and pATARC4-SAS, respectively. Lines were ranked firstly on the basis of the number of transgene copies and secondly on the basis of Arc5a expression levels (indicated as percentage of total seed protein). Values of protein levels are followed by the S.D. Results of quantification of mRNA steady state levels in developing siliques are presented as the molar ratio of *arc5-I* mRNA to *at2S* mRNA. ND, not determined.

[a]In case of multiple copies, the organization (when known) is indicated between brackets, IRR = inverted repeat over the right border, IRL = inverted repeat over the left border, TR = tandem repeat.

[b]Because of the limited number of seeds available, quantification was only performed once and no S.D. could be calculated.

For many plant systems, it would be interesting to repress or inactivate a gene that encodes a major endogenous storage protein and thereby elevate the levels of other endogenous or transgene-derived proteins. One way to achieve this goal is by conventional breeding, as was, for example, demonstrated in *P. vulgaris* lines in which genetic removal of the major seed storage protein (phaseolin) by introducing a null allele resulted in an important increase of Arc accumulation levels [24]. Because null alleles are not available for most storage proteins, the antisense technology could provide an interesting alternative as has been shown here and previously [3–5]. This could have major applications in the generation of transgenic plants of which seeds possess an improved nutritional quality or synthesize pharmaceutically or industrially interesting products.

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