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Tobacco ribosomal DNA spacer element stimulates amplification and expression of heterologous genes

Nikolai Borisjuk[1,2], Ludmyla Borisjuk[2,3], Slavko Komarnytsky[1], Sofia Timeva[1], Vera Hemleben[4], Yuri Gleba[5], and Ilya Raskin[1]*

[1]Biotech Center, Foran Hall, Cook College, Rutgers University, 59 Dudley Rd., New Brunswick, NJ 08901-8520, USA. [2]Phytomedics, Inc., 2245 Route 130, Suite 103, Dayton, NJ 08810, USA. [3]Present address: Institute of Plant Genetics, Correns Str. 3, Gatersleben D-06466, Germany. [4]Center of Plant Molecular Biology, University of Tuebingen, Auf der Morgenstelle 28, Tuebingen D-72076 Germany. [5]Institute of Cell Biology and Genetic Engineering, Zabolotnogo St., 148, Kyiv, DSP-22, 252650, Ukraine. Corresponding author (raskin@AESOP.RUTGERS.EDU).

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Here we show that the *cis*-acting genetic element *aps* (amplification-promoting sequence), isolated from the nontranscribed spacer region of tobacco ribosomal DNA (rDNA), increases the level of expression of recombinant proteins. Transgenic tobacco plants, transformed with expression cassettes containing the herbicide-resistant acetolactate synthase (hr-ALS) gene or the green fluorescent protein (GFP) gene fused to the *aps* sequence, had greater levels of corresponding messenger RNAs (mRNAs) and proteins compared to transformants lacking *aps*. Analysis of transgenic plants showed that *aps* increased the copy number and transcription of the adjacent heterologous genes and, in the case of hr-ALS, enhanced the herbicide resistance phenotype. Both the increased transgene copy number and enhanced expression were stably inherited. These data provide the first evidence that the *aps* sequence can be used for gene amplification in transgenic plants and possibly other multicellular organisms.

Keywords: gene amplification, enhanced transcription, protein expression, transgenic plants, recombinant proteins

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A high level of transgene expression is a requirement for any economically effective protein production system. Plants are recognized as an attractive system for the commercial production of valuable recombinant proteins, whereas low levels of transcription often limit the expression of heterologous genes in plants. High-yielding expression systems in plants, which use recombinant viruses[1] or transformed chloroplasts[2], rely on the high copy number of introduced genes per cell. Both of these systems, however, have a number of technical and environmental limitations. Gene amplification (increase in gene copy number on the chromosome) may provide an effective alternative strategy for the production of desired proteins in plants.

Gene amplification was first observed for dihydrofolate reductase (DHFR) in cultured murine cells selected to grow on increased concentration of a cytotoxic compound methotrexate[3]. DNA amplification is common for many multicellular species and occurs naturally during the ontogenesis[4], carcinogenesis[5], and in in vitro-cultivated cells[6]. A number of specific *cis*-acting DNA elements that regulate gene amplification have been identified in mammalian cells. Many of these elements, such as a DNA fragment that induces amplification in response to 5-bromo-deoxyuridine[7], mouse amplification-promoting sequence *muNTS* (ref. 8), and the repetitive genomic DNA element HSAG (ref. 9), share common structural features. The A+T-rich tracks and repeated sequences homologous to yeast ARS (autonomously replicating sequence) consensus sequence, are among such structures[10,11]. When included into transfected vectors, these sequences have been shown to amplify adjacent genes up to 800 times and increase gene expression[9,12,13]. Despite some generational instability, gene amplification is recognized as one of the most successful strategies for achieving high levels of protein production in cultivated mammalian cells[14].

Gene amplification in plants occurs during evolution[15], in vitro plant culture[16], and in response to metabolic stress[17]. However, until this report, no amplification-stimulating DNA elements from plants have been identified thus far. Moreover, directed amplification of heterologous genes has never been achieved in transgenic multicellular organisms such as plants.

Results and discussion

Identification of potential *aps* in tobacco rDNA. Earlier investigation of the nuclear genomes of tobacco somatic hybrids and potato plants regenerated from protoplasts using rDNA as a genome-specific RFLP marker, showed significant molecular rearrangements in the rDNA locus[18]. These rearrangements included appearance of new variants of rDNA repeats not present in the parental genomes. Analysis of the novel rDNA repeats in the *Nicotiana tabacum* × *Atropa belladonna* hybrid[19] showed that they were formed as a result of spontaneous length increase in the intergenic spacer (IGS) region of a single rDNA repeat unit followed by the amplification of this new rDNA variant (Fig. 1A). The observed increase in rDNA copy number suggested that the IGS region of rDNA (ref. 20), fully sequenced in potato[21] and tobacco[22,23], may contain regulatory elements that promote DNA amplification.

While searching for the regulatory DNA element responsible for gene amplification, we identified an A+T-rich region in the rDNA IGS regions of tobacco (Fig. 1B) and potato (data not shown) that showed homology to the A+T-rich domain of a cloned mouse rDNA element *muNTS1,* previously shown to stimulate DNA amplification in mammalian cells transfection experiments[8]. In addition, both tobacco and potato A+T-rich domains contained nine (Fig. 1C) and eight (data not shown) regions, respectively, with a mini-

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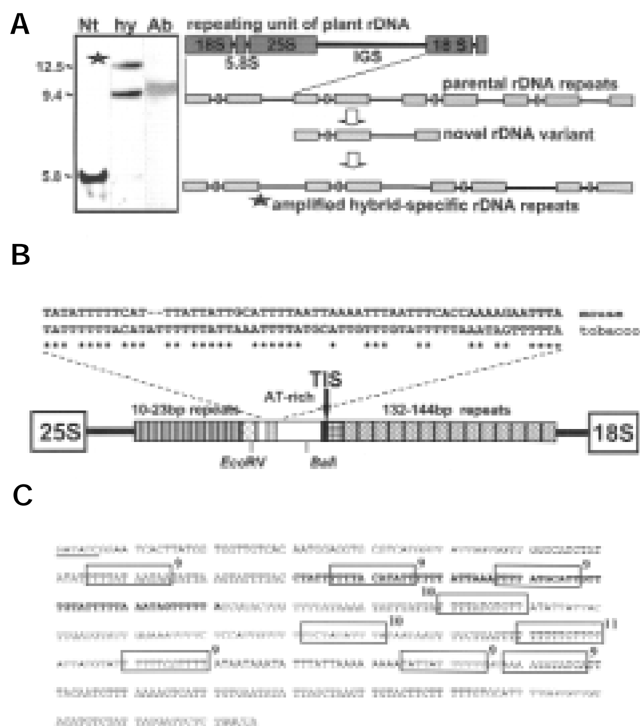

Figure 1. Identification and molecular organization of the tobacco *aps*. (A) Identification of the novel rDNA repeats variant in the genome of *Nicotiana tabacum* (Nt) × *Atropa belladonna* (Ab) somatic hybrid (hy) and a schematic representation of the rDNA amplification (right). Total DNA was digested with *Eco*RV and hybridized to 25S rRNA gene-specific probe[19]. Numerals indicate molecular sizes of hybridization fragments in kilobases. 18S, 5.8S, and 25S indicate, correspondingly, coding sequences for 18S, 5.8S, and 25S rRNA. (B) Molecular structure of the tobacco rDNA intergenic spacer (IGS). Nucleotide sequence alignment shows domain of the highest homology between mouse *muNTS1* sequence and tobacco A+T-rich region. 25S and 18S represent ribosomal genes. TIS, rDNA transcription initiation site. (C) Nucleotide sequence of the IGS fragment used as an *aps* in plant transformation experiments. *Eco*RV and *Bal*I restriction sites are underlined. Bold letters show the sequence with the highest homology to *muNTS1*. Boxes correspond to a unique sequence where nine near matches to the yeast 11 bp ACS core were found. Numbers above each box indicate the number of matches.

mum 9/11 match to the highly conserved 11 bp core consensus sequence (ACS) of the *Saccharomyces cerevisiae* ARS (A/T)TTTAT(A/G)TTT(A/T). This sequence was located within the origins of DNA replication in several eukaryotes, including plants[24]. Mouse *muNTS1* and several other mammalian amplification-promoting elements[7,10,25] also contained the ACS consensus sequences, which suggests that ARS-like elements are required for gene amplification in both mammals and plants.

***aps* increases herbicide resistance in transgenic tobacco.** To determine whether the tobacco rDNA A+T-rich spacer element promotes amplification of heterologous genes in transgenic tobacco plants, a 440 bp long fragment between the *Eco*RV and *Bal*I restriction sites of the IGS region containing ACS-like sequences (Fig. 1C) was incorporated in plant transformation vectors containing expression cassettes for plant hr-ALS (ref. 26) and jellyfish GFP (refs 27, 28). Tobacco transformants, produced as described earlier[28], were analyzed and compared to control transformants containing the same expression cassettes lacking the putative *aps*. The hr-ALS expression cassette was composed of the *Arabidopsis thaliana* ALS promoter, the mutant *A. thaliana* ALS coding sequence conferring resistance to the sulfonylurea herbicides (i.e., Pursuit), and a terminator (Fig. 2A). All first-generation *aps*-containing and control transformants were selected on medium containing 1 μM of Pursuit. DNA and RNA isolated from randomly chosen hr-ALS and hr-ALS-*aps* regenerants were subjected to Southern and northern analysis (Fig. 2B). Comparative analysis of hr-ALS and hr-ALS-*aps* groups showed a statistically significant (*t*-test, $P < 0.05$) increase in the copy number of *hr-ALS* genes and corresponding mRNA in transformants containing *aps*. Hr-ALS transformants contained 3.2 copies of the gene on average ± 1.76 s.d., whereas in the hr-ALS-*aps* group the average copy number was 5.8 ± 2.78 s.d., with all plants containing more than three copies of the gene (Fig. 2B, C). Hr-ALS-*aps* plants also accumulated on average 4.5 times more hr-ALS mRNA than plants without *aps*.

Increased expression of the hr-ALS gene controlled by *aps* resulted in a striking enhancement of the herbicide resistance phenotype in a rooting test[26]. We planted 40 hr-ALS-*aps* tobacco transformants, 38 hr-ALS transformants, and 12 wild-type tobacco plantlets and vegetatively propagated them on Murashige–Skoog (MS) medium supplied with 5, 10, 15, and 20 μM Pursuit. On the medium containing 10 μM of the herbicide, 42% of hr-ALS-*aps* plants and only 5% of hr-ALS plants survived (Table 1). On the medium containing 15 and 20 μM herbicide, 22 and 12% of the hr-ALS-*aps* transformants, respectively, were able to grow, whereas none of the hr-ALS survived these concentrations (Fig. 2D). These data suggest that *aps*-induced gene amplification caused major enhancement of the transgenic trait, that is, the herbicide resistance phenotype.

Inheritance of amplified ALS gene expression in T_1 seed generation. Whereas the enhanced gene expression was stable in the vegetatively propagated tobacco, we tested whether this feature is inherited through the meiosis and is transmissible through seeds. Five transgenic hr-ALS-*aps* plants (lines 16, 17, 21, 22, and 25, Fig. 2B) that expressed high amounts of hr-ALS-specific mRNA and grew on 15 μM or 20 μM herbicide in agar medium were used for the analysis. All lines produced normal seeds when grown in the greenhouse. In order to select transgene from homozygous nontransgenic plants, seeds were germinated on kanamycin-containing agar medium. Close to 75% of all T_1 seeds geminated and produced plants, indicating that the introduced expression cassette was inherited as a single dominant mendelian locus. Comparative DNA and RNA analysis of four individual seed-derived plants for each line (Fig. 2E) showed that in four out of five lines, both *hr-ALS* gene copy number and expression were maintained at the same level as in the original vegetatively propagated T_0 plants. Despite the fact that the high copy number of the hr-ALS genes in T_1 plants of line 22 was the same as in the parent plant, mRNA pattern differed significantly from the uniform high expression in the other four lines. The decline in the mRNA level in the reference parental line as compared to that obtained six months earlier (Fig. 2B, lane 22), can be attributed to the methylation and partial gene silencing of the transgene that occurred after the original mRNA analysis. The absence of any detectable hr-ALS mRNA in two out of four T_1 plants may be caused by the homozygous transgene silencing[29].

Table 1. Numbers of transgenic tobacco plants rooting on herbicide-containing medium

Herbicide concentration (μM)	pNB-ALS-*aps*	pNB-ALS	Non-transgenic plants
0	40	38	12
1	40 (100%)	38 (100%)	0 (0%)
5	25 (63%)	5 (13%)	0 (0%)
10	17 (42%)	2 (5%)	0 (0%)
15	7 (22%)	0 (0%)	0 (0%)
20	5 (12%)	0 (0%)	0 (0%)

Numbers in parentheses indicate the percentage of plants able to root and grow on each concentration of herbicide.

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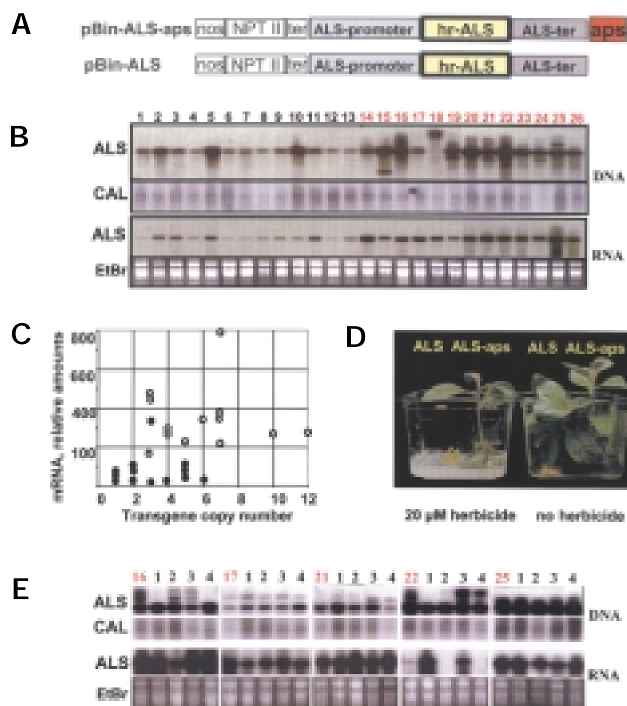

Figure 2. Expression of the *hr-ALS* gene in ALS and ALS-*aps* transformants. (A) Schematic representation of ALS and ALS-*aps* expression cassettes used for tobacco transformation. nos-NPT II-ter, neomycin phosphotransferase expression cassette conferring kanamycin resistance; hr-ALS, *A. thaliana* mutant gene conferring sulfonylurea herbicide resistance; *aps*, *Eco*RV–*Bal*I fragment of the tobacco rDNA spacer region (see Fig. 1B,C). (B) Comparative analysis of hr-ALS-specific DNA and mRNA in hr-ALS (lanes 1–13) and hr-ALS-*aps* (lanes 14–26) tobacco transformants. Top panel: Southern hybridizations of *Xba*I-digested DNA with ALS-specific probe (ALS) and calreticulin-specific (CAL) probe as a single-copy gene reference. Bottom panel: northern analysis of hr-ALS-specific mRNA (ALS), and ethidium bromide-stained gel (EtBr) showing RNA loading. (C) Distribution of hr-ALS mRNA in hr-ALS (●) and hr-ALS-*aps* (○) transformants as related to *hr-ALS* gene copy number. (D) Representative hr-ALS (left plant) and hr-ALS-*aps* (right plant) tobacco plants grown in MS medium containing 20 µM Pursuit herbicide (left box) and in MS medium without herbicide (right box). (E) Inheritance of amplified ALS gene in T_1 generation. Lanes 16, 17, 21, 22, and 25 represent original vegetatively propagated plants corresponding to transgenic lines in (B). Lanes 1–4 refer to individual plants of sexually produced T_1 generation of the original transgenic line.

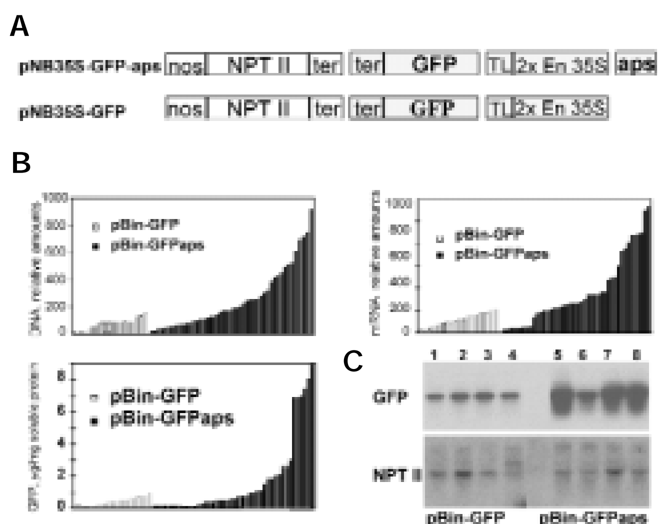

Figure 3. Expression of GFP in GFP and GFP-*aps* transformants. (A) Schematic representation of GFP and GFP-*aps* expression cassettes used for the generation of transgenic plants. nos-NPT II-ter, Neomycin phosphotransferase expression cassette conferring kanamycin resistance; 2xEn 35S-TL-GFP-ter, GFP expression cassette[27,28]. (B) Comparative analysis of GFP-specific DNA, mRNA, and protein in population of GFP (□) and GFP-*aps* (■) transformants. Southern and northern blots were quantified using ImageMaster VDS software (Amersham Pharmacia Biotech), and the protein amount determined by ELISA. (C) Effect of *aps* on GFP gene expression in transgenic lines with low copy number of GFP expression cassette. Northern hybridization of mRNA isolated from GFP (lines 1–4) and GFP-*aps* (lines 5–8) plants with GFP (top) and NPT II (bottom) probes. The same filter containing 10 µg of RNA was hybridized to ^{32}P-labeled GFP probe, stripped, and rehybridized to the NPT II-specific probe.

***aps* enhances expression of GFP.** To further demonstrate the gene expression-promoting effect of *asp*, GFP controlled by the cauliflower mosaic virus (CaMV 35S) promoter[28] was used to construct pNB35S-GFP and pNB35S-GFP-*aps* vectors (Fig. 3A), which were transformed into tobacco. As revealed by comparative Southern hybridization, all regenerated transformed plants lacking *aps* contained 1–2 copies of the GFP gene, whereas 60% of GFP-*aps* transformants contained 3–12 copies of the GFP gene (average 4.8 copies ± 2.44 s.d.) and significantly larger (t-test, $P < 0.05$) amounts of the GFP mRNA (Fig. 3B; see original Southern and northern hybridization as Supplementary Figure 1 on the Web Extras page of *Nature Biotechnology* Online). mRNA expression level is not always proportional to the copy number. A similar situation was reported for mammalian gene amplification systems[12], indicating the dominant effects of the integration sites on the efficiency of expression. Whereas Figure 3A depicts only one orientation of GFP expression cassette relative to the *aps*, we tested also the opposite orientation, as in the case of ALS-*aps* (Fig. 2A), with the *aps* adjacent to the 3′ end of the GFP cassette. It appears that *aps* orientation has no effect on the transgene copy number or expression. The data confirming inheritance and analyzing chromosomal organization of amplified GFP expression cassette are presented as Supplementary Fig. 2 on the Web Extras page, *Nature Biotechnology* Online.

GFP-*aps* plants contained more GFP protein than GFP transformants, as determined by enzyme-linked immunosorbent assay (ELISA) analysis using anti-GFP monoclonal antibodies[28]. The average amount of GFP in the GFP plants was 0. 46 µg/mg total soluble protein, whereas the GFP-*aps* plants averaged 2.28 mg GFP/mg total soluble protein. Protein expression was not directly proportional to gene copy number; nevertheless, by plotting copy number against mRNA and protein expression levels we obtained a positive correlation between DNA copy number and gene expression.

***aps* as an enhancer of transcription.** In some GFP-*aps* plants containing only one or two copies of the GFP gene, an unexpectedly high mRNA expression compared with GFP transformants with the same gene copy numbers was observed. In these plants the GFP-*aps* cassette directed two to five times higher expression of GFP mRNA in comparison to the GFP transformants without *aps* (Fig. 3C). To the contrary, the expression of the *NPT II* gene (neomycin phosphotransferase; see Fig. 2A), situated in the same expression cassette but at a greater distance from *aps*, was similar in both GFP and GFP-*aps* plants. Therefore, it is likely that *aps* only affects transcription of immediately adjacent regions of DNA. Transcription activation by *aps* is not particularly surprising, in that it is located upstream of the transcription initiation site in tobacco rDNA IGS. In several plant species this region of IGS has been shown to contain an intrinsic DNA bending center, which is implicated in the regulation and/or initiation of transcription, replication, and recombination by facilitating specific protein–DNA interactions[30]. Bent DNA regions are characterized by the A+T-rich sequences such as putative bent DNA motives[31], and/or scaffold-attached regions (SAR)[32], which are present in multiple copies in the *aps*. Experiments demonstrated that

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aps behaves as a typical DNA bent structure in a polyacrylamide gel, assayed at different temperatures to determine such regions. Excised, *aps* migrated anomalously as compared to a reference DNA fragment (data not shown). Thus, *aps* is likely to stimulate the expression of heterologous genes by increasing both their copy number and transcription.

Conclusions. In contrast to the situation with mammals, the molecular mechanisms of gene amplification in plants are poorly understood. We have identified and functionally characterized the *aps* from tobacco rDNA. This element significantly increases the copy number and expression of adjacent heterologous genes, acting in both upstream and downstream position. The *aps* was successfully tested in combination with two expression cassettes composed of plant ALS gene/ALS promoter, and jellyfish GFP gene/viral CaMV 35S promoter. The effect of *aps* is likely due to a combination of its structural features such as its 80% A+T content, repetitive ARS core elements, DNA bending, and SAR-related sequences. All of these could be involved in formation of protein–DNA complexes that initiate and regulate nearby gene amplification and transcription, similar to *aps* functions at its original position upstream of the transcription initiation site of rDNA. The enhancing effect of the *aps* element is stable in the T_1 generation in terms of both copy number and expression. Therefore, the genetic element, *aps*, may be used as a new tool to enhance gene expression in transgenic plants created for agricultural uses and molecular farming.

Experimental protocol

Construction of expression vectors. Construction of the pNB-GFP-*aps* and pNB-ALS-*aps* vector began from the plasmid Nt4-19 containing central A+T-rich region of tobacco IGS, which showed sequence homology to the mouse amplification-promoting element *muNTS1* (ref. 12) by BLAST search. An *Eco*RI–*Bal*I fragment (nucleotides 1,642–2,081 of the tobacco rDNA intergenic spacer region, European Molecular Biology Laboratory accession no. Y08422) was cut out from Nt4-19 and placed between *Eco*RI and *Sma*I sites of a general plant transformation vector pBin19 (ref. 33), resulting in the pBin-*aps*. The GFP expression cassette[27,28] and the hr-ALS cassette[26] were cut out with *Hin*dIII and *Xba*I, respectively, and ligated into the corresponding sites of pBin-*aps*, to produce plant transformation vectors pNB-ALS-*aps* and pNB35S-GFP-*aps*. For the control transformation vectors pNB35S-GFP and pNB-ALS, the same procedure was repeated with the original pBin19 plasmid. For the gel mobility assay the *Eco*RI–*Bal*I *aps* fragment was cloned into *Eco*RI–*Sac*I of plasmid Litmus-28 (New England BioLabs, Beverly, MA), resulting in Lit-*aps*, and digested with *Eco*RI–*Stu*I to cut out the *aps*.

Plant transformation and cultivation. Tobacco plants (*Nicotiana tabacum* L., cv. Wisconsin) were transformed by standard *Agrobacterium* leaf disk co-cultivation and selected on 100 mg/L kanamycin MS medium as described[28]. The rooting test for hr-ALS transformants was performed as described[26] on the agar MS medium supplemented with 0, 1, 5, 10, 15, and 20 μM Pursuit herbicide (American Cyanamid Company, Princeton, NJ).

Comparative DNA, mRNA, and protein analysis. Total genomic DNA was isolated using Phytopure plant DNA extraction kit (Nucleon Biosciences, Lanarkshire, UK). After complete digestion with restriction enzymes, DNA fragments were separated by agarose gel electrophoresis, transferred to Hybond-N^+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ), and hybridized to ^{32}P-labeled DNA fragment specific for GFP, ALS, NPT II, and calreticulin, a tobacco endoplasmic reticulum protein, used as an internal single-copy gene standard[34]. The Southern blot images were quantitatively evaluated using ImageMaster VDS software (Amersham Pharmacia Biotech). Relative GFP and ALS hybridization signals for each plant were adjusted according to calreticulin hybridizations, and the lowest GFP or ALS radioactivity count equated to a single gene copy per genome. The isolation and hybridization analysis of RNA were conducted as described[28], and the quantification of hr-ALS and GFP mRNA expression was performed as described above for DNA blots. Amounts of GFP protein extracted from plant tissues were determined by ELISA analysis using anti-GFP monoclonal antibodies as described earlier[28].

Note: Supplementary information can be found on the Nature Biotechnology *website in Web Extras (http://www.biotech.nature.com/web_extras).*

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