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The upstream Sal repeat-containing segment of *Arabidopsis thaliana* ribosomal DNA intergenic region (IGR) enhances the activity of adjacent protein-coding genes

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Abstract

The sequence containing 'upstream Sal repeats' (USR) from the *Arabidopsis thaliana* ribosomal DNA intergenic region (IGR) was tested for its influence on the *in vivo* activity of nearby protein coding genes. On average, the presence of the IGR fragment leads to a four-fold increase in the expression of a reporter gene, β-glucuronidase, under control of the strong CaMV 35S promoter. With the help of the site-specific cre-lox recombination system, we have also obtained pairs of transgenic lines with or without the USR-containing fragment, both integrated at the same chromosomal position. Results with these transgenic lines, which contain an NPT II (kanamycin resistance) gene under control of the *nos* promoter as a test gene, confirmed the results obtained with the CaMV 35S-driven GUS gene. Moreover, they show that the IGR sequence can oppose tendencies of gene silencing. We hypothesize that the described effect relates to features of the chromatin structure in the proximity of the upstream Sal repeats.

Introduction

Ribosomal genes comprise a multigene family typically arranged as a tandem array at a few genetic loci called nucleolus-organizing regions (Mélèse and Xue, 1995; Shaw and Jordan, 1995; Paule, 1998; Jacob and Ghosh, 1999; Carmo-Fonseca *et al.*, 2000; Olson *et al.*, 2000; González-Melendi *et al.*, 2001). While sequences coding for rRNA are well conserved, those between the coding regions, particularly in the intergenic region (IGR) which connects the transcription units, are not conserved. In spite of this poor sequence conservation, one can describe prototypic features for the IGR architecture: close to the 3′ end of the large rRNA gene, there is a transcription termination site for RNA polymerase I (Mason *et al.*, 1998). A transcript spanning 18S, 5.8S and 26–28S rRNA initiates at a site close to the 18S coding region. This putative RNA polymerase I promoter has been found to be surprisingly compact both in animals and in plants (Hemleben and Zentgraf, 1994; Moss and Stefanovsky, 1995). As a consequence, a typical IGR contains several kilobases of sequence in addition to signals for transcription initiation and termination. A deeper understanding of these IGR sequences is beginning to emerge for the budding yeast *Saccharomyces cerevisiae* (Davis *et al.*, 2000; Kobayashi *et al.*, 2001; Wai *et al.*, 2001), but experiments in other organisms usually lack a comparable degree of sophistication.

In almost all species, IGR sequences have, at a position upstream of the transcription initiation site, numerous short blocks of sequences occurring as tandem repeats. There is, in general, no sequence similarity between repeats from different species, implying that these sequences evolve rapidly. In the *A. thaliana* IGR, a 2.26 kb region contains the so-called upstream Sal repeats (USR) (Gründler *et al.*, 1991; Wanzenböck *et al.*, 1997). In two different assay systems, no ef-

fect of upstream sequences on the RNA polymerase I core promoter activity could be detected (Doelling and Pikaard, 1995; Wanzenböck *et al.*, 1997). The fact that blocks of repeats seem to be a constant feature in plant IGR sequences (Hemleben and Zentgraf, 1994) suggests nonetheless an associated functionality. We therefore sought to find an experimental set-up which might help to reveal a potential function for these repeats. Here we show that transcription by RNA polymerase II, a process with the potential to respond to the chromatin environment, is influenced by the nearby presence of a USR-containing fragment.

Materials and methods

Plant genotypes, selection conditions

Arabidopsis thaliana ecotype Col-0 was used for transformation. A line expressing the *cre* gene under control of the CaMV 35S promoter (Line 1998.2; No-0 background, Kan resistance marker) was kindly provided by Dr I. Chou, Plant Gene Expression Center, Albany, NY). Selection for kanamycin resistance was carried out on plates containing 20 mg/l kanamycin. Unless stated otherwise, the concentration of hygromycin in growth media was 25 mg/l. Presence of a *cre* gene (Sternberg *et al.*, 1986) was assayed by PCR with primers *cre* up (CCTGATCCTGGCAATTTCGGCTAT) and *cre* dn (CGCGGTCTGGCAGTAAAAACTAT). Presence or absence of USR sequences was tested by PCR using oligos flanking the USR insert (primer 1 of Figure 6, AACCGCAGCAGGGAGGCAAACAAT, and primer 4 of Figure 6, CAGAAACCCGCGGCTGAGTGGCT), and with oligos that bind to flanking regions and to USR sequences (primer 1 together with primer 2 of Figure 6, GTTGAAATCGTCGACCAGGACC, and primer 3 of Figure 6, CCAAGTAATAACATTTAACCTC, together with primer 4).

Plant transformation

Standard conditions for transformation were applied. To obtain callus material, the method of Valvekens *et al.* (1988) was adopted, except that hygromycin resistance was used as a selection marker. Callus material was taken from callus-inducing medium, and put on agar medium containing 1 mg/l benzylaminopurine and 0.1 mg/l naphthyl acetic acid for further growth. To obtain transgenic plants, the vacuum infiltration method of Bechthold *et al.* (1993) was applied.

Vector construction

An *Xho*I-*Eco*RI fragment (the *Eco*RI recessed end was filled in by use of Klenow fragment of *Escherichia coli* DNA polymerase I) from *Arabidopsis thaliana* rDNA (600–2857 of accession number X15550; Gründler *et al.*, 1989) was inserted between *Xba*I (filled in) and *Xho*I sites of vector pSK+ to give pUSR. A *Hin*dIII linker (New England Biolabs, Schwalbach, Germany) was inserted into the *Xba*I site to give plasmid pUSR1H. A *Hin*dIII-*Xho*I fragment from pUSR1H was inserted between *Hin*dIII and *Sal*I sites of the binary plant vector pBIB-HYG (Becker, 1990) to give pBIB USR. A *Hin*dIII fragment from plasmid pRT104GUS, which contains the *E. coli uidA* β-glucuronidase-encoding gene under the control of the CaMV 35S promoter and terminator (a gift of R. Töpfer, Max-Planck-Institut für Züchtungsforschung, Cologne, Germany), was inserted into the unique *Hin*dIII site of pBIB USR to give vector BG+USR. The orientation of the insert in the latter plasmid is such that the promoter is close to USR sequences (Figure 1). Vector BG+USR was digested with *Sma*I and partially with *Hin*dIII. After Klenow fill-in and religation, the ensuing vector was called BG-USR.

To obtain a vector with the USR sequence flanked by lox sites, a partial *Eco*RI-*Hin*dIII insert fragment from vector pGU.US (Tinland *et al.*, 1994; a kind gift of B. Hohn, Friedrich-Miescher-Institut, Basel, Switzerland) was ligated into *Eco*RI-*Hin*dIII-digested vector pΔHV (Bachmair *et al.*, 1990) to give pΔHVG/US. pUSR was modified by insertion of the 34 bp *lox* sequence on both sides with double-stranded oligonucleotides (annealed oligos SloxE1, CTAGATATAACTTCGTATAATGTATGCTATACGAAGTTATT and SloxE2, AATTAATAACTTCGTATAGCATACATTATACGAAGTTATATCTAG for one side, and SloxA1, TCGAGATATAACTTCGTATAATGTATGCTATACGAAGTTAATCTA and SloxA2, GTACCTCTAGAATAACTTCGTATAGCATACATTATACGAAGTTAT for the other side). After modification of the ensuing clone pSK2loxUSR by insertion of *Hin*dIII linkers on either side to obtain vector pSK2HloxUSR, a *Hin*dIII fragment from pSK2HloxUSR was inserted into pΔHVG/US to give vector pΔHVloxUSR.

Determination of GUS activity in cell extracts

The method was adapted from Martin *et al.* (1992) and Wilson *et al.* (1992). Fresh callus material (20–40 mg) was homogenized in 200 μl extraction buffer (50 mM

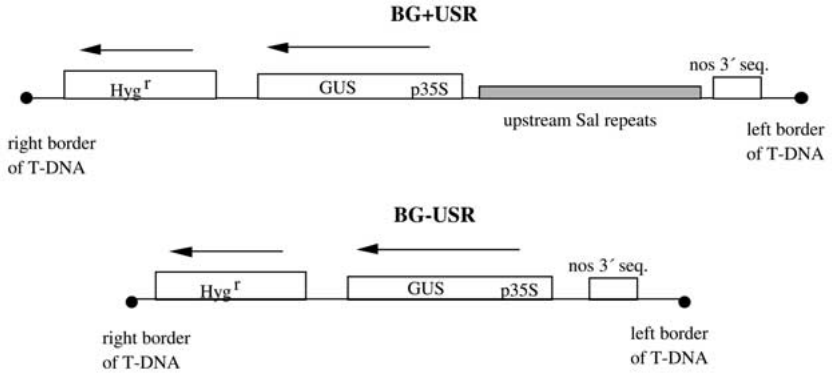

Figure 1. T-DNA constructs used to assay the influence of a USR (upstream Sal repeat) containing fragment on RNA polymerase II mediated gene expression. Top, construct BG+USR. Bottom, construct BG–USR. GUS, reading frame of *E. coli* β-glucuronidase; p35S, promoter of cauliflower mosaic virus 35S transcript; Hyg^r, hygromycin phosphotransferase gene under control of the *Agrobacterium* nopaline synthase promoter; nos 3′ seq., termination region of nopaline synthase gene.

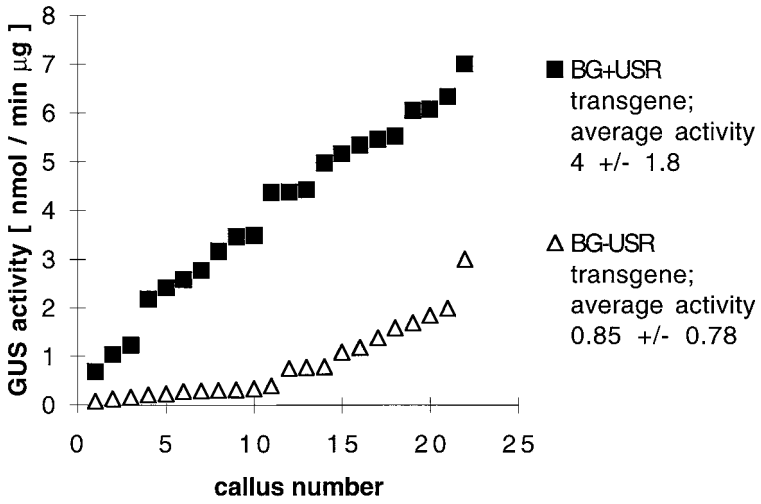

Figure 2. GUS activity in transgenic callus. A total of 22 *Arabidopsis* calluses transformed with construct BG+USR (closed squares), and 22 calli containing a BG–USR transgene (open triangles), were randomly selected and used for GUS activity measurements. Calluses were numbered in order of activity. On average, construct BG+USR results in a more than fourfold higher activity than construct BG–USR.

sodium phosphate pH 7, 10 mM 2-mercaptoethanol, 10 mM EDTA, 0.1% Triton-X 100, 0.1% SDS) with an electric homogenizer (IKA Ultra Turrax, IKA, Staufen, Germany). Care was taken to cool the material on ice throughout the procedure. Cell debris was removed by centrifugation (15 min, 4 °C, 10 000 × *g*). Thereafter, protein concentration was determined with a BioRad protein assay reagent (BioRad, Vienna, Austria) as recommended by the manufacturer. A 1–5 μl portion of the protein extract was incubated with 450 μl GUS buffer (50 mM sodium phosphate pH 7, 5 mM DTT, 1 mM EDTA, 2 mM *p*-nitrophenyl-β-D-glucuronide (Sigma-Aldrich, Vienna, Austria)) at 37 °C. Depending on the speed of the reaction, samples were taken every 5 to 15 min. For that purpose, 100 μl of the sample was withdrawn and added to 800 μl of 0.4 M Na_2CO_3. Absorbance at 405 nm was determined and used to calculate enzyme activity (molar extinction coefficient 18 000).

GUS staining of leaves

Leaves from soil-grown homozygous plants were fixed (2% paraformaldehyde, 100 mM sodium phosphate pH 7, 1 mM EDTA, 0.1% Triton X-100) and vacuum-infiltrated with 100 mM sodium phosphate pH 7, 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 1 mM X-Gluc and 0.1% Triton X-100. The reaction was stopped by

Table 1. GUS activity and transgene copy number of randomly chosen calluses.

Callus number /transgene construct	Enzyme activity (nmol min^{-1} μg^{-1}) mean value[a] ± SD	Hybridization signal ratio GUS / Pol II[b]	Estimated transgene copy number
1 / BG–USR	0.07 ± 0.04	1.85	4
2 / BG–USR	0.12 ± 0.08	4.64	11
3 / BG–USR	0.15 ± 0.09	1.10	3
4 / BG–USR	0.20 ± 0.14	2.97	7
5 / BG–USR	0.23 ± 0.05	3.98	10
6 / BG–USR	0.27 ± 0.18	1.96	5
7 / BG–USR	0.29 ± 0.20	3.08	8
8 / BG–USR	0.30 ± 0.13	2.19	5
9 / BG–USR	0.31 ± 0.18	2.89	7
10 / BG–USR	0.33 ± 0.13	2.39	6
11 / BG–USR	0.39 ± 0.42	2.41	6
12 / BG–USR	0.75 ± 0.79	2.10	5
13 / BG–USR	0.77 ± 0.50	1.50	4
14 / BG–USR	0.78 ± 0.40	0.70	2
15 / BG–USR	1.08 ± 1.05	1.16	3
16 / BG–USR	1.18 ± 0.11	0.68	2
17 / BG–USR	1.38 ± 0.40	0.90	2
18 / BG–USR	1.58 ± 0.91	1.39	3
19 / BG–USR	1.69 ± 1.32	0.99	2
20 / BG–USR	1.84 ± 0.11	0.82	2
21 / BG–USR	1.99 ± 0.95	0.79	2
22 / BG–USR	3.00 ± 3.12	7.72	19
1 / BG+USR	0.68 ± 0.28	0.95	2
2 / BG+USR	1.04 ± 0.99	2.68	6
3 / BG+USR	1.23 ± 0.11	7.04	15
4 / BG+USR	2.18 ± 0.60	0.51	1
5 / BG+USR	2.42 ± 1.86	2.74	6
6 / BG+USR	2.58 ± 1.43	0.84	2
7 / BG+USR	2.77 ± 0.36	0.63	2
8 / BG+USR	3.17 ± 1.19	0.86	2
9 / BG+USR	3.46 ± 0.12	0.72	2
10 / BG+USR	3.49 ± 1.74	0.79	2
11 / BG+USR	4.37 ± 1.84	0.52	1
12 / BG+USR	4.38 ± 1.34	0.70	2
13 / BG+USR	4.43 ± 3.81	0.79	2
14 / BG+USR	4.98 ± 1.80	0.47	1
15 / BG+USR	5.17 ± 2.44	0.58	1
16 / BG+USR	5.34 ± 1.00	0.48	1
17 / BG+USR	5.47 ± 0.84	0.36	1
18 / BG+USR	5.52 ± 1.85	0.64	2
19 / BG+USR	6.05 ± 1.64	0.44	1
20 / BG+USR	6.07 ± 1.70	0.64	2
21 / BG+USR	6.33 ± 0.73	0.73	2
22 / BG+USR	7.00 ± 5.12	1.23	3

[a]GUS assays were carried out with three different samples for each transgenic callus, except for callus 20/BG–USR, where only two activity tests were used to calculate the mean value.
[b]For each callus, the transgene band (probe homologous to β-glucuronidase), and the genomic band (probe homologous to a subunit of RNA polymerase II) of a DNA gel blot experiment (cf. Figure 3) were quantified by phosphorimager analysis. The ratio of the intensities is listed.

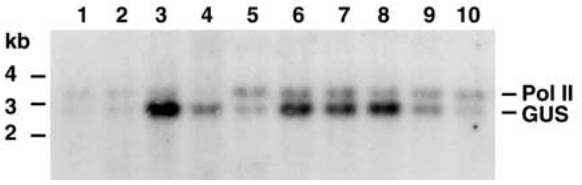

Figure 3. Determination of transgene copy number. Callus DNA was CsCl-purified, digested with *Eco*RI and subjected to gel blot analysis. Filters were hybridized to detect the GUS transgene, and the largest subunit of RNA polymerase II as an internal standard. Lanes 1–3, DNA from calluses 17/BG-USR, 19/BG-USR and 7/BG-USR, respectively, of Table 1 and Figure 2 was used for digestion; the callus of lane 4 was not used for calculations due to the high hybridization background and is not listed in Table 1; lanes 5–10, calluses 21/BG-USR, 11/BG-USR, 6/BG-USR, 4/BG-USR, 13/BG-USR and 20/BG-USR, respectively. GUS, position of band from transgene; Pol II, position of band from endogenous gene. Position of molecular weight marker bands is indicated to the left (size in kb).

one wash in H_2O and subsequent incubation in 70% ethanol (adapted from Martin *et al.*, 1992).

DNA isolation

Fresh callus material (2–5 g) was frozen in liquid N_2 and ground to a fine powder. The powdered material was added to 15 ml of extraction buffer (100 mM Tris-HCl pH 8, 50 mM EDTA, 0.5 M NaCl, 10 mM 2-mercaptoethanol) supplemented with 3 ml 10% SDS, which was heated to 65 °C. The slurry was kept at 65 °C for 20–180 min. After addition of 7 ml potassium acetate (3 M), the sample was gently mixed and kept on ice for 20–60 min. Insoluble debris was removed by centrifugation (4 °C, 35 000 × *g*, 15 min). After filtering the supernatant through nylon gaze, 10 ml of 2-propanol were added, and the sample was placed at −20 °C overnight. Nucleic acids were collected by centrifugation (4 °C, 35 000 × *g*, 15 min), the pellet was washed with ethanol (70%), and dried. After suspension in 1 ml TE buffer (10 mM Tris pH 8, 1 mM EDTA) by warming to 65 °C, RNase A (20 μg) was added, and the sample was incubated at 30 °C for 30 min. Excess carbohydrates were precipitated by addition of 0.03 volumes of potassium acetate (3 M) and 0.1 volume of ethanol, 15 min incubation on ice, and a centrifugation step (4 °C, 10 000 × *g*, 15 min). The supernatant was further purified by addition of 0.5 ml phenol/chloroform/isoamyl alcohol (25:24:1), inversion of the sample, and centrifugation (10 min, 5000 × *g*). The supernatant was used for a CsCl gradient as described (Ausubel *et al.*, 1987).

Figure 4. Leaves from transgenic plants carrying construct BG+USR (left column), and BG–USR (right column), respectively, were stained under identical conditions to visualize differences in GUS expression levels. For activity values in cell extracts, see text.

DNA gel blot hybridization and quantification

For DNA gel blots, 10 μg of CsCl-purified DNA was incubated overnight with 100 U restriction enzyme (Roche Diagnostics, Vienna, Austria; Fermentas, St. Leon-Roth, Germany). An aliquot of the DNA was run on a minigel to assess the completion of digestion. Gel electrophoresis and transfer were carried out according to standard protocols (Ausubel *et al.*, 1987) on nylon filters (Amersham Hybond N, Amersham Pharmacia, Little Chalfont, UK). DNA fragments for labelling and hybridization were generated by PCR reaction (primers CTGTGGGCATTCAGTCTGGATC and GGGATAGTCTGCCAGTTCAGTTC for GUS, and CGTGGCTTGACTCCTCAAGAGTTC and GGACCTCACAAATTCAAAGTCCTC for the largest subunit of RNA polymerase II), or by excision from plasmids. Hybridized and washed filters were first exposed to X-ray film (Kodak X-Omat AR, Kodak, Rochester, USA) and then analysed with a phosphorimager (Molecular Dynamics STORM 840, equipped with Image Quant processing software; Molecular Dynamics, Krefeld, Germany). Bands of interest were framed and counted. A frame of the same size in a blank area served to subtract a background value.

RNA analysis

RNA was prepared with the Qiagen RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. A 10 μg portion of total RNA was applied per lane in gel blot experiments. The RNA was size-separated on formaldehyde containing agarose gels with a MOPS-containing running buffer and transferred onto nylon membranes as described (Ausubel *et al.*, 1987). Detection and quantification of RNA was done as described for DNA gel blots.

Results

In an effort to define a biological role for sequences of the *Arabidopsis* IGR, we tested whether transcription by RNA polymerase II, a process that is sensitive to the chromatin environment, is influenced by the nearby presence of a DNA fragment that contains USR sequences. To that end, two plant transformation vectors were constructed (Figure 1). In the backbone of the binary plant transformation vector pBIB-HYG (Becker, 1990), a GUS gene was inserted under control of the CaMV 35S promoter. Next to this reporter gene, one of the two vectors contains the 2.26 kb USR region from *A. thaliana* ribosomal DNA (for details, see Materials and methods). The other vector does not contain this sequence, but is otherwise identical and serves as a control. Close to the left T-DNA border, both vectors contain a piece of 'buffer' DNA (a nopaline synthase terminator region) which may decrease the influence of sequences outside the T-DNA on gene expression (Breyne *et al.*, 1992).

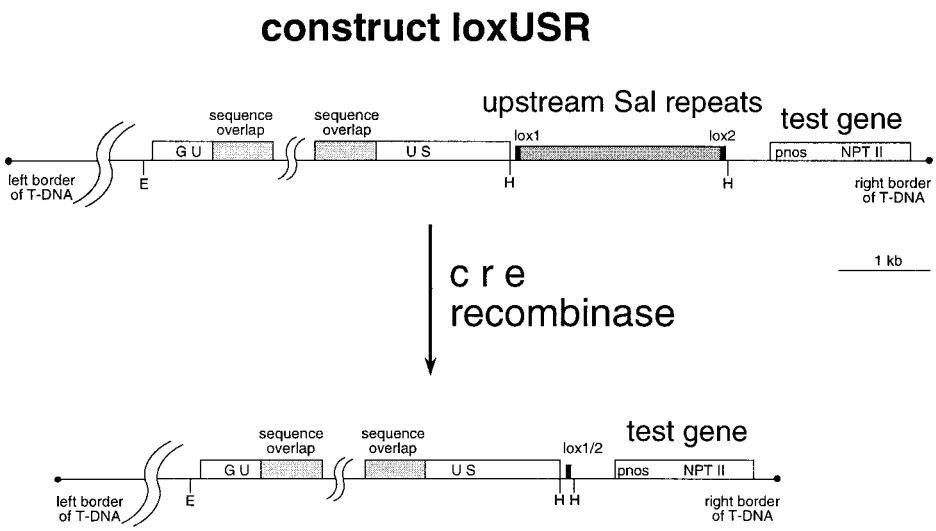

Figure 5. Strategy for comparison of transgenes +/− USR-containing fragment, integrated at an identical chromosomal position. Construct loxUSR contains the fragment flanked by lox sites, adjacent to a neomycin phosphotransferase II test gene driven by a *nos* promoter. Crossing of plant lines containing this construct to a line that expresses cre recombinase removes the USR sequence, and, in addition, reduces the number of T-DNA copies in case of a tandem T-DNA arrangement. An interrupted GUS (GU–US) gene is not exploited in this work and may serve to measure recombination frequencies in the vicinity of the IGR fragment.

Transformation of *Arabidopsis* with these two vectors was carried out by the root transformation method (Valvekens *et al.*, 1988) to obtain callus material. During root transformation, care was taken not to put a strong selection pressure on the transgenic material during the first time in tissue culture. This was achieved by using only 5 mg/l hygromycin in the media until calluses had reached a diameter of ca. 2–3 mm.

GUS activity was determined for a randomly chosen array of 22 calluses for each of the two vector constructs. Parts of tissue were removed from three different sectors of the same callus. Assays were carried out in triplicate and normalized to the protein content. Results of this series of experiments are depicted in Figure 2 and in Table 1. On average, the construct BG+USR allows more than four-fold higher gene expression than the control construct, BG–USR.

We were particularly interested in knowing whether the presence of the test fragment has any influence on the integration pattern of the T-DNA. For instance, the effect of USR sequences could correlate with differences in the copy number of integrated transgenes. Using the same callus material that served to obtain samples for enzyme assays, we sought to determine transgene copy numbers. DNA from transgenic callus material was isolated, digested and used for gel blot analysis. Digestion was carried out with an enzyme that excises the reporter transgene from T-DNA, so that all copies of the GUS transgene present in the genome of a transgenic callus gave the same size fragment. The DNA gel blot was then hybridized with a GUS probe. As an internal control, a probe from the gene for the largest subunit of RNA polymerase II (Nawrath *et al.*, 1990) was also included in the hybridization. Assuming complete digestion of the plant DNA, we expected two bands of different size, one for the transgene(s), and one for the cellular gene.

Figure 3 shows a typical result. To unambiguously assign the bands to the respective genes, the filter was washed and re-hybridized with the GUS probe only. After quantification of the band intensities, the ratio of transgene band to endogenous band was calculated for each lane as a measure of transgene copy number. The lowest ratio of GUS to Pol II signal was assumed to represent one copy of T-DNA. The higher values corresponded to 2, 3, 4, 5, 6, 7, 8, 10, 11, 15 and 19 copies (see Table 1). Because both transgenes, BG+USR and BG–USR, hybridize to the GUS probe with the same intensity, all calluses with similar intensity ratio carry a similar transgene copy number. The number of integrated T-DNA copies in BG+USR is not significantly different from BG–USR calluses. We therefore conclude that the enhancing effect of the USR-containing fragment is not caused by copy number differences.

Using the *in planta* transformation method (Bechthold *et al.*, 1993; G. Cardon, personal communication), we made transgenic lines to find out whether the increased expression level caused by the IGR fragment can also be observed in whole plants. Plants with integrated constructs BG+USR and BG–USR, respectively, were generated and selfed to obtain homozygous lines. Figure 4 shows stained leaves from four plants of each group. Because of the considerable difference in activity between the two groups, the GUS activity of plants without USR-containing fragment is barely visible after the short staining period employed to underscore differences among the BG+USR plants. Leaf extracts from five lines transformed with BG+USR, and four lines transformed with BG–USR were taken for GUS measurements. In the T_2 generation, the BG+USR group had an average activity of 1.47±0.92 nmol/min per μg (mean ± SD) in young leaves, whereas the mean activity in the BG–USR group was 0.11±0.15. This represents a more than thirteen-fold difference in GUS activity. Interestingly, we found that GUS activity of lines containing BG+USR was almost identical between different generations, whereas activity in BG–USR lines differed considerably from generation to generation (we observed both increases and decreases; data not shown). We therefore suggest that the USR sequence has a stabilizing effect on the expression of the test gene. Most importantly, the positive effect of the IGR fragment on gene expression is apparent both in callus material, and in transgenic plants, and is inheritable over at least four generations.

The experiments described above do not rule out the possibility that the USR-containing fragment influences T-DNA integration by favouring integration into chromosomal loci that allow a high transgene expression level. For this and other reasons, it seemed of interest to compare transgenes with and without test sequence, which were integrated at the very same chromosomal position. We used the cre-lox recombination system in a strategy depicted in Figure 5 to make such a comparison. T-DNA vector pΔHVloxUSR contains the IGR fragment between two lox sites. The test gene of the construct is a kanamycin resistance gene driven by the *nos* promoter. Use of a different promoter (pnos vs. CaMV 35S), a different test gene (neomycin phosphotransferase II vs. GUS) and a different detection method (mRNA quantification vs. enzyme activity measurement) should serve to find out whether the effect is independent of any particular RNA polymerase II driven promoter, and whether it involves changes in transcriptional activity.

We obtained over 30 *Arabidopsis* lines transformed with the pΔHVloxUSR construct. Competitive PCR between a single-copy genomic sequence and T-DNA sequences was used to identify lines with one or only a few copies (data not shown). These lines were crossed to a line expressing cre recombinase. Application of cre recombinase would not only remove the test fragment from the T-DNA, but would also remove entire T-DNA copies if present in a tandem array. Furthermore, incomplete T-DNA copies with only one lox site may retain (part of) the test sequence even in the presence of cre.

F_2 plants from the cre/loxUSR crosses were crossed to Col-0 wild type, and segregants which had lost cre, but contained the test gene, were selected by PCR. These lines were investigated for the absence of the USR-containing fragment using PCR analysis with a number of diagnostic primers (Figure 6). Homozygous lines that had tested as expected in all screens were selected for further work. Next, quantitative DNA gel blot hybridization was carried out using both a restriction enzyme/DNA probe combination entirely contained within the T-DNA (*Pae*I/*Hin*dIII), and a combination that allowed us to detect fragments with flanking DNA (*Eco*RI/*Hin*dIII). DNA gel blot experiments of the former type are best for copy number determination, whereas the latter type also confirmed that pairs of lines had been assigned correctly. The gel blot results also confirmed genetic data that plants of interest had single locus T-DNA insertions. Furthermore, both blots confirmed that cre recombinase-induced rearrangements were indeed restricted to recombination between lox sites. Figure 7 shows the result of this experiment. In addition to autoradiography, the blots were used for phosphorimager analysis. Three lines (numbers 5, 16 and 32) were of particular interest, because they had a single-opy, complete T-DNA insert, and the USR sequence had been cleanly removed by the cre recombinase. Another line, number 42, had three copies of the test gene in one locus before action of cre recombinase and one copy (with all USR sequences removed) after 'loxing'.

Determination of mRNA levels for the pnos-driven NPTII transgene in each pair of transgenic lines was carried out by gel blot experiments followed by exposure to X-ray film (Figure 8), and by phosphorimager analysis (Figure 9). Similar to the previous results with callus material and transgenic plants carrying the BG+USR and BG–USR transgenes, the USR se-

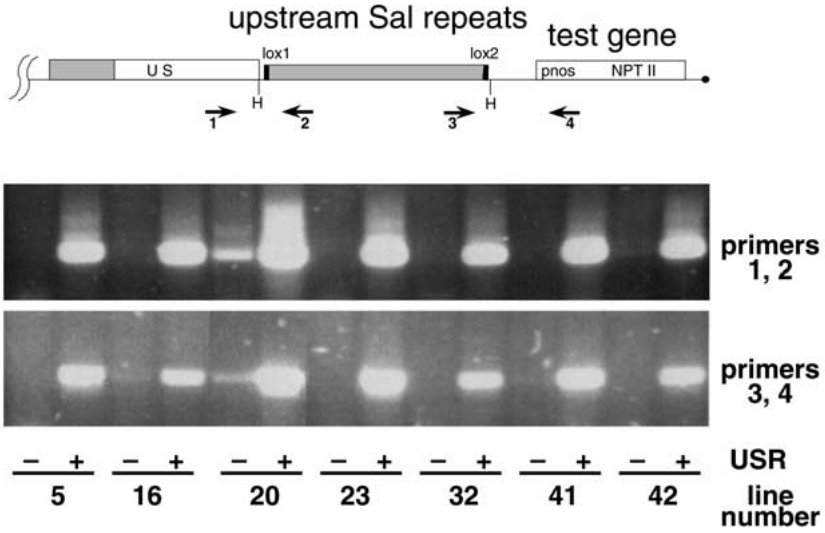

Figure 6. PCR analysis to assess removal of the USR-containing test fragment from different transgenic lines. Cre apparently did not remove all USR copies from line 20, which was therefore not used in further comparisons.

quence brings an increase in activity. Line 5+USR has a three-fold higher mRNA level than line 5–USR, and line 32+USR has a two-fold higher level than line 32–USR. Line 16+USR has roughly the same NPTII mRNA level as line 16–USR. Furthermore, we find lines 42+USR and 42–USR of particular interest. Line 42+USR has three copies of the transgene in one genetic locus. After 'loxing', one copy without USR sequences was left. While there is, statistically, a decrease in activity with increasing copy number, or no correlation between copy number and activity (Vain *et al.*, 1999), we find that the higher-copy line 42+USR has an at least three-fold higher mRNA level than the line with a single-copy transgene integrated at the same position, 42–USR. The decrease in mRNA level can be visualized by a loss of kanamycin resistance (Figure 10). Because marker transgenes that do not suffice to confer a resistance phenotype are conventionally called 'silenced', one can ascribe an anti-silencing effect to the USR-containing fragment.

Discussion

In this work, we investigate the role of a segment from the non-coding intergenic region of *Arabidopsis thaliana* ribosomal DNA. When placed next to a protein-coding (RNA polymerase II transcribed) gene, the ribosomal sequence, which contains the highly repetitive so-called upstream Sal repeats (USR), increased gene expression and stabilized the expression level of adjacent genes (Figures 2, 4, 8, 9 and 10, and data not shown). The effect is apparently independent of the orientation of the IGR fragment (constructs BG+USR and pΔHVloxUSR have the sequence in opposite orientation relative to the test gene). However, the orientation may have an influence on the magnitude of the enhancing effect. The experiments shown suggest that the effect is not restricted to a single promoter, but works both with the strong CaMV35S promoter, and with the weaker *nos* promoter.

It should be emphasized that the enhancing effect of ribosomal sequences on RNA polymerase II transcription is probably not correlated with so-called ribosomal enhancers, i.e. sequences which enhance RNA polymerase I transcription. For instance, Ghosh *et al.* (1994) investigated the effects of the '130 bp repeat element' of rat IGR. Presence of this sequence enhances RNA polymerase I transcription in rat cells, but not RNA polymerase II transcription in a transient assay system of hamster cells. Conversely, the *Arabidopsis* USR-containing fragment investigated in this work enhances RNA polymerase II transcription, but an effect on RNA polymerase I transcription could not be detected (Wanzenböck *et al.* 1997). The two results concerning *Arabidopsis* IGR sequences can be reconciled if the effect of these sequences is somewhat redundant within the rDNA, so that deletion of the USR-containing fragment has only a minor effect. In that case, the isolated presence of a segment from

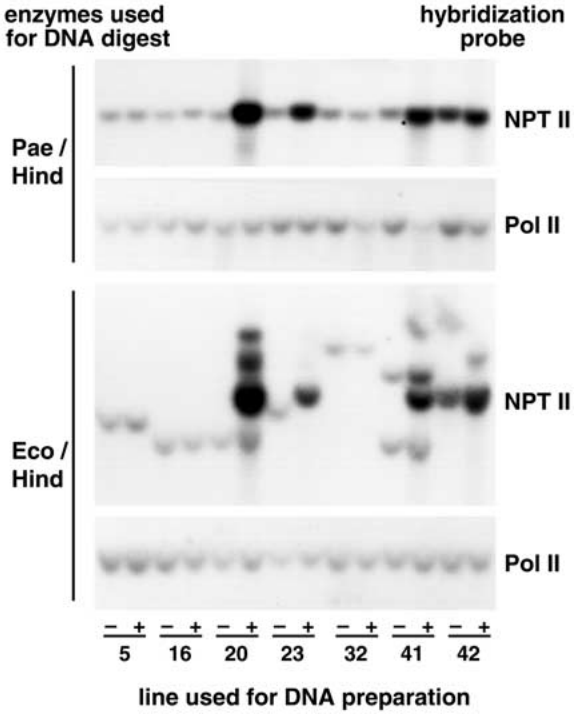

Figure 7. DNA gel blot experiment to assess the copy number of loxUSR transgenes before and after cre activity. Filters were probed sequentially with a probe from the NPT II gene, and the RNA polymerase II large subunit gene as a control. The *Pae*I/*Hin*dIII enzyme combination excises the detected fragment from T-DNA, whereas enzyme combination *Eco*RI/*Hin*dIII allows visualization of fragments which contain flanking DNA. Lines 5, 16 and 32 have single-copy T-DNA inserts both before and after cre-mediated recombination. In the other lines, cre-mediated recombination leads to changes in copy number, so that effects on gene expression are more difficult to interpret. For further discussion, see text.

the IGR in a different context, as exemplified in this work, is better suited to reveal potential functions. The enhancement effect of the IGR fragment in a different sequence context, but not in their natural environment, has an interesting parallel in the budding yeast, where biological effects of a presumed ribosomal enhancer region were most difficult to detect in their natural gene environment (Wai *et al.*, 2001).

Two of the constructs compared in this work, BG+USR and BG–USR, differ in that omission of the USR-containing fragment places the GUS reporter construct closer to the T-DNA border. Breyne *et al.* (1992) have shown that this change in itself may influence transgene expression in tobacco. However, both construct pΔHVloxUSR and 'loxed' genomic derivatives devoid of the USR-containing fragment have the test gene close to the right T-DNA border at an identical position, and the distance to the left border is longer than 10 kb both in lines with, and in those without IGR sequence. The result of this second series of comparisons therefore strongly suggests that the increase in gene expression is due to the USR-containing ribosomal spacer sequence, and not to the changed distance of a test gene from the T-DNA border.

The data presented suggest that the USR-containing fragment does not influence the structure of integrated transgenes. In particular, the experiments with randomly chosen calluses showed that the IGR sequence does not lead to a higher or lower copy number of the integrated T-DNA. Experiments with randomly chosen inserts, however, do not rule out the possibility that the change in average activity is due to a biased distribution of insertion sites, as opposed to an increase in activity for any given individual T-DNA insertion site. Such a biased distribution could occur if, for instance, the test fragment would favour integration of T-DNA into particularly active chromatin, or if it would selectively decrease the activity of transgenes with an intrinsically low activity. In the latter case, the 'low-activity' transformation events would not survive the selection process. The experiments with construct loxUSR (cf. Figures 9 and 10) indicate that the USR-containing fragment changes the gene expression of transgenes irrespective of whether the chromosomal location is intrinsically permissive, or rather inhibitory to high gene expression levels (cf. lines 5 and 32 of Figure 9).

While our interest focuses on repetitive sequences called upstream Sal repeats (USR), the test fragment also contains other sequences from IGR. In particular, a transcription termination site for RNA polymerase I is present. RNA polymerase I termination sequences have been shown to influence polymerase I transcription initiation in mouse (Längst *et al.*, 1998) and in yeast (Morrow *et al.*, 1993). However, the effect requires specific and/or precise interactions with protein factors which are probably not present on RNA polymerase II promoters. Furthermore, no similar effect was reported for plants. Another feature of the USR test fragment is the presence of AT-rich stretches between two blocks of relatively GC-rich repeats. Sequences with similarity to the transcription initiation site, so-called spacer promoters, are also contained. The latter sequences can act as *in vivo* transcription initiation sites for RNA polymerase I in a transient assay system (Doelling *et al.*, 1993), but a positive influence on rRNA levels has not been found (Doelling and Pikaard, 1995; Wanzenböck *et al.*, 1997). At present, we cannot rule out the possibility that any

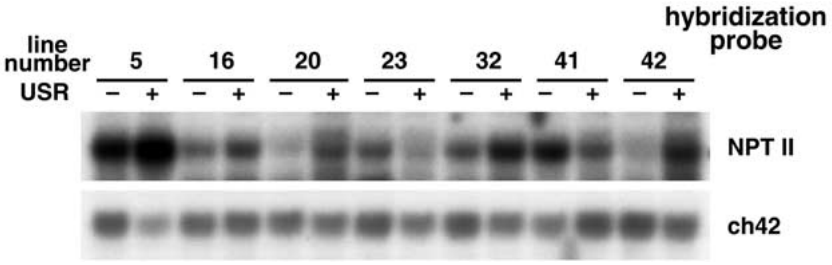

Figure 8. RNA gel blot experiment to measure the expression level of the NPT II test gene. After autoradiography, the filter was used for quantification by phosphorimager. The chlorata 42 (ch42) mRNA served as a loading control.

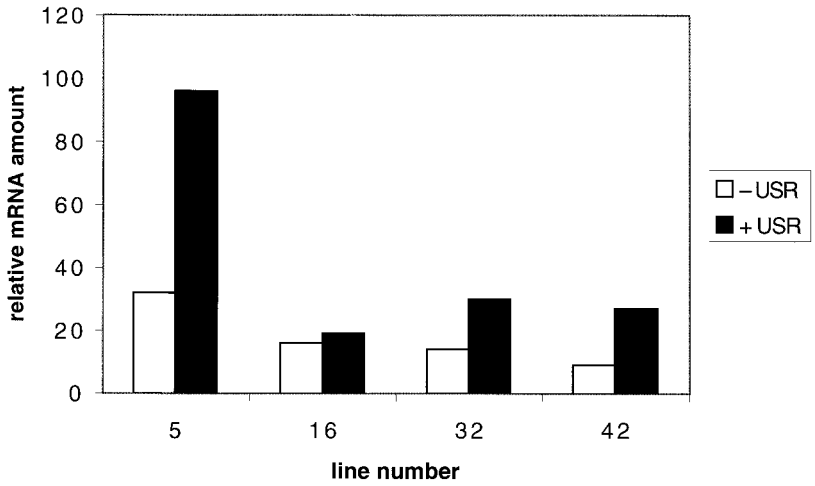

Figure 9. Differences in gene expression between lines with and without USR sequence next to the NPT II test gene, as obtained by phosphorimager analysis of the gel blot shown in Figure 8. Lines 5, 16 and 32 contain a single copy of the transgene before and after 'loxing', whereas line 42 had 3 copies before, and one copy after cre-mediated recombination. The relative mRNA amount listed reflects the ratio of expression of the test gene and the internal control gene, ch42.

of these additional sequences, present next to the upstream Sal repeats, contributes to the effect described in this work. Using sub-fragments of the IGR segment employed in this investigation, it may be possible in the future to understand the influence of each of these sequences.

It is interesting to compare our results to those of Borisjuk *et al.* (2000). Using vector constructs similar to BG+USR and BG–USR, Borisjuk *et al.* found an enhancing effect of tobacco IGR sequences on RNA polymerase II transcription in tobacco. While the magnitude of the effect is comparable to our findings, Borisjuk *et al.* (2000) also found that their IGR segment enhances copy number of the integrated transgenes. Interestingly, we obtain in our assay a larger number of transgenic calluses carrying BG+USR when compared to calluses with BG–USR, if the selection with hygromycin is kept at 25 mg/l throughout the experiment (the experiments of Table 1 and Figures 2 and 3 used an initial hygromycin concentration of only 5 mg/l). Our interpretation of this finding is that many transformation events with BG–USR do not allow a sufficiently high transcription rate for manifestation of resistance at early stages of growth. It is known that multi-copy insertions are more likely to allow only modest expression of transgenes, so that many of these 'unselectable' T-DNA insertion events may actually represent multi-copy inserts. Our data and those of Borisjuk *et al.* suggest that ribosomal sequences can also enhance transcription from multi-copy inserts, so that many more multi-copy arrays could confer a sufficient level of resistance and thus show up as transgenic material. More generally, any sequence that enhances gene expression both on single, and on multi-copy inserts, may skew the distribution of T-DNA copy number after selection for transgene expression.

One of the 'loxUSR' lines described in this work may be relevant for the assessment of the IGR fragment's effects on multi-copy arrays. Line 42–USR has a single-copy insert that allows only a modest level

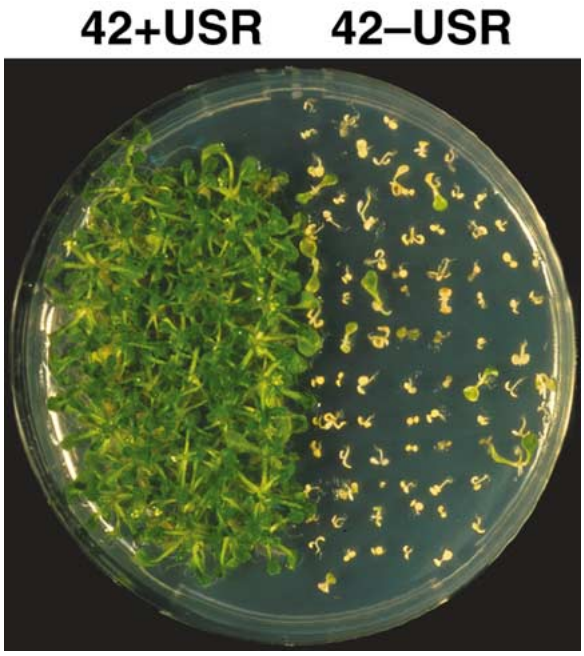

Figure 10. Loss of kanamycin resistance in line 42 after removal of a USR-containing fragment by cre recombinase. Line 42–USR cannot grow on plates with 20 mg/l kanamycin, indicating that its transgene is 'silenced'. In contrast, line 42+USR can grow on the same plates, indicating that the test fragment ensures stable and sufficient expression of the NPT II test gene.

of test gene expression. The level is insufficient for growth on plates containing 20 mg/l kanamycin and may serve to document the influence of the chromosomal environment on gene expression at the site of T-DNA insertion in this line. Line 42+USR has the same test gene at the identical position. It is, however, adjacent to the USR-containing fragment, and two additional T-DNA copies are also present, resulting in a three copy array. While multiple copies usually lead to a decrease in activity, the three-copy arrangement of line 42+USR has a three times higher expression level than line 42–USR (Figure 9) and can grow on kanamycin plates (Figure 10). As mentioned before, this result suggests an anti-silencing effect of the USR sequence. rDNA is not entirely resistant to gene silencing effects. Silencing effects can occur in crosses between closely related plant species and result in epigenetic down-regulation of ribosomal transcription in one of the two combined genomes (this phenomenon is called nucleolar dominance; for review, see Pikaard, 2000). However, mechanisms must exist to ensure that the tandem array of rDNA is not silenced during normal growth. Our experiments indicate that *Arabidopsis* IGR sequences may contribute to the resilience of rDNA against repeat-induced gene silencing.

In an attempt to interpret the positive effect of the USR-containing fragment on RNA polymerase II transcription, we are currently focusing on properties of genetic loci that are summarized under the term 'chromatin structure'. We hypothesize that IGR sequences such as the upstream Sal repeats are, in their native environment, involved in the establishment of ribosomal chromatin. Based on our experiments, an IGR fragment may be used as a portable *cis* element capable of establishing a similar chromatin structure when placed elsewhere in the genome. The effect may be additive with existing chromatin environments. If this is the case, the mode of action of this sequence is distinct from that of so-called scaffold attachment regions (SARs), which were also found to enhance RNA polymerase II transcription (Allen *et al.*, 1996; Ülker *et al.*, 1999; Vain *et al.*, 1999). It is, in our opinion, also unlikely that non-ribosomal sections of the genome, when noticed for transcription enhancing properties (Ott and Hansen, 1996), have a similar mode of action.

The transgenic lines with transgenes integrated at the same position were also created for future experiments. Firstly, differences in chromatin structure caused by the test sequence can be assessed, e.g. by nuclease sensitivity assays, with high precision. Secondly, an attempt can be made to determine whether the location of the transgene within the nucleus is influenced by the USR-containing fragment. In particular, randomly integrated, complete rDNA repeat units on a T-DNA were previously found to favour nucleolar localization (Wanzenböck *et al.*, 1997). The lines obtained in this work allow to assess whether an IGR fragment alone has a similar effect. Thirdly, it is known that recombination within the rDNA is much lower than in euchromatin (Copenhaver *et al.*, 1995). The presence of a recombination substrate, which was previously used to monitor intrachromosomal recombination (Swoboda *et al.*, 1994), may help to investigate the influence of the test sequence on recombination. Irrespective of future directions, our data, as well as those of Borisjuk *et al.* (2000), indicate that sections from rDNA can help to create transgenic plants with increased and stable expression levels of protein coding genes.

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