Silencing of a plant gene by transcriptional interference

Boris Hedtke and Bernhard Grimm*

Institute of Biology/Plant Physiology, Humboldt University, Philippstr.13 Building 12, 10115 Berlin, Germany

Received October 24, 2008; Revised March 5, 2009; Accepted March 30, 2009

ABSTRACT
Integration of foreign DNA into eukaryotic genomes results frequently in a total or partial loss of gene function, caused by the interruption of indispensable structures of the gene itself. Using T-DNA insertions in Arabidopsis we screened for mutants with deregulated chlorophyll precursor accumulation in etiolated seedlings. A mutant designated rfd1 (red fluorescent in darkness) with increased protochlorophyllide accumulation showed a fluorescent phenotype that was associated with a lack of transcript initiation from the AtRibA1 promoter situated downstream of the integrated T-DNA. Complementation experiments confirmed rfd1 to be a knockout phenotype. Comparison with two SALK insertion lines bearing T-DNA integrations in the 5′ UTR of AtRibA1 demonstrated that the insertion event in rfd1 itself does not explain the complete lack of transcript initiation. A 35S tetrameric enhancer sequence present on the rfd1 T-DNA causes the overaccumulation of a large polycistronic transcript originating inside the T-DNA. This 5.5-kb RNA runs over the downstream situated AtRibA1 promoter, which was shown by 5′RACE analyses to be consequently silenced. Hence, a transcription process that starts upstream and overlaps AtRibA1 blocks the initiation at the AtRibA1 promoter in rfd1. This regulatory mechanism has recently been introduced in yeast as transcriptional interference and is described here for the first time in a plant system.

INTRODUCTION
T-DNA insertions have become a common tool in plant mutant analysis and proved extremely useful to unravel functions by forward as well as by reverse genetic approaches. The interruption of gene function by the insertion of T-DNA results in the generation of knockout mutants and enables screens of mutant collections for the identification of unknown genes contributing to specific cellular functions as well as the analysis of the function of known genes (1). Functional knockout or knockdown of gene products are expected if the T-DNA is inserted into the coding region of a gene. However, T-DNA insertions hit introns or up- and downstream regions of a gene of interest as well as intergenic DNA stretches (2). Such insertion events often fail in generating phenotypical changes. Different types of T-DNA constructs have been used to expand the possibilities of T-DNA transformation as a tool in plant biology. Activation tagging and entrapment tagging are the most prominent strategies to explore the genomic neighborhood of the integrated DNA (3,4).

Recent work in yeast (5) demonstrated that transcription of the non-coding upstream region is able to silence the Saccharomyces cerevisiae SER3 gene by preventing the binding of transcriptional activators necessary for recruiting RNA polymerase II. This mechanism was designated ‘transcriptional interference’ to indicate that it is the process of transcription itself that interferes with the initiation of mRNA synthesis from downstream promoters. Using a forward genetic screen to identify mutants in the regulation of tetrapyrrole biosynthesis, we identified a T-DNA mutant line with a specific recessive phenotype. An enhanced promoter located inside the T-DNA region caused a strong expression extending into the genomic neighborhood of the integration site. This apparently resulted in a drastic perturbation of transcription initiation from the downstream-situated Arabidopsis gene. Our findings suggest transcriptional interference as the mechanism responsible for gene silencing.

MATERIALS AND METHODS
Plant material and growth conditions
An Arabidopsis mutant collection of 30000 independent lines transformed with plasmid pWAS (kindly provided by T. Altman, Gatersleben) was used in the initial screen. Described SALK mutants (2) were obtained from the NASC (Nottingham). Seeds were surface-sterilized by incubation in Meliseptol (Braun, Melsungen) for 3 min. and subsequently rinsed four times with distilled water. Seeds were sown on agar plates containing 0.5×

*To whom correspondence should be addressed. Fax: +49 30 20936337; Email: bernhard.grimm@rz.hu-berlin.de

© 2009 The Author(s)
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.0/uk/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.
Murashige and Skoog (MS) salts including vitamins (Duchefa, Haarlem) and 0.05% MES, pH 5.7. Plates for growth under etiolated conditions were incubated for 3 h in the light and subsequently wrapped in light-tight plastic foil. Following incubation for 2 days at 4°C, the plates were transferred for 5 days to room temperature. For analyses of light-grown rfd1 mutants, surface-sterilized seeds were sown on MS plates containing 2% sucrose.

Microscopy and protochlorophyllide measurement

Etiolated seedlings were inspected using a Leica stereomicroscope with fluorescence unit and a blue excitation filter set (excitation 470/40 nm, barrier 515 nm). For the determination of protochlorophyllide (PChlide) amounts, samples of 20 seedlings were harvested, steamed over a boiling water bath for 2 min and frozen in liquid N2. Homogenization using an eppendorf pistil was done in acetone/0.1N H2O (9/1). Pchlide was determined in a Hitachi F4500 fluorescence spectrophotometer using excitation/emission wavelength of 440/632 nm, respectively.

Nucleic acid extraction and PCR

Following the collection of etiolated seedlings under the fluorescence microscope, RNA of seedlings was extracted using Trisure (Bioline GmbH, Luckenwalde) according to the manufacturer’s instructions. cDNA synthesis was performed from 1 μg total RNA with random hexamer primers and RibLock RNase Inhibitor and RevertAid Reverse Transcriptase (Fermentas, St. Leon-Rot). Semi-quantitative RT-PCR used 2–18 ng of reverse transcribed total RNA, Mangotaq DNA polymerase (Bioline GmbH, Luckenwalde) and primer pairs F3 aacgggagtatcag and tuaR acagcatgaaatggatacgg (At5g19780, tua5), respectively.

5’ RACE reactions were performed using RNA purified with RNeasy Plant Mini Kit (Qiagen, Hilden) from rfd1 mutants and Arabidopsis wild-type plants grown on agar plates. SALK mutant lines 036891 and 094736 were selfed in the light and subsequently wrapped in light-tight plastic foil. Following incubation for 2 days at 4°C, the plates were transferred to a new tube and frozen in liquid N2. Larger amounts of RNA were isolated following the method described in (7).

Identification of insertion sites

T-DNA flanking sequences were determined using the protocol of Strizhov et al. (8). PCRs were performed using pWA5 left border primers LB1 ttataaatcaacctggagctacatatttt and LB2 ttgtaacttctttctttctttattta. Amplified fragments were sequenced with LBseq, gctaca cacaactcctattt and R2 ctgctattggtgagac (see Figure 1E and G). For genetic complementation experiments, At5g64300 including promoter sequences was amplified using primers F2 cc gggacatctactaag and R2 ctgctattggtgagac (see Figure 1E). The 5-kb product was inserted into binary vector pBinAR following removal its 35S promoter. The resulting plasmid pRIB was used to transform Arabidopsis rfd1 mutants by vacuum infiltration.

Northern and southern blot analyses

RNA separated on formaldehyde-containing gels was blotted on Hybond N membranes (GE Healthcare, Munich) and hybridized to PCR fragments that were radioactively labeled with α32P-dCTP (Hartmann Analytic, Braunschweig) by Decaprime labeling kit (Fermentas, St. Leon-Rot) using standard protocols (9). Hybridization signals were detected using a Bio-Rad PhospholImager. Southern blotting was performed according to standard protocols (9) using a 760-bp PCR fragment amplified with primers SB_F gccacactatcagact and SB_R gccacactatcagact from pWA5 plasmid DNA as probe.

RESULTS

Mutant screen and initial analysis of rfd1

The precursor of all tetrapyrroles, 5-aminolevulinic acid (ALA), and its synthesis in dicotyledonous plants is strongly reduced in the dark (10). A screen for mutants affected in control of ALA biosynthesis was performed using a transgenic population of 30000 independent Arabidopsis T-DNA insertion lines transformed with plasmid pWA5 (kindly provided by T. Altmann, Gatersleben). Mutants that do not sufficiently repress ALA synthesis under etiolated growth conditions...
accumulate excessive amounts of the chlorophyll precursor Pchlide, which is converted to chlorophyllide in a light-dependent enzymatic step. Several mutants showing a characteristic red fluorescent phenotype under etiolated growth conditions (red fluorescent in darkness, rfd) were identified.

Pigment extraction from fluorescing rfd1 mutant seedlings revealed an ~8-fold increase in accumulated protochlorophyllide. These highly fluorescent seedlings exhibited normal skotomorphogenetic growth with wild-type length hypocotyl and closed cotyledons after 5–7 day’s growth on agar plates in the dark (Figure 1A). When etiolated rfd1 mutants were transferred to light, the over-accumulated chlorophyll precursor caused photooxidative damage and the respective seedlings subsequently died. Hence, the phenotype in the dark strongly resembles the flu mutant described by Meskauskine et al. (11).

However, when grown in constant light or dark–light cycles, the mutant showed completely white cotyledons (Figure 1D). True leaves were similarly bleached under normal light conditions although growth under dim light (5–20 μM photons m⁻² s⁻¹) results in a slight greening. On sucrose-supplemented media rfd1 developed inflorescences and sets flowers, but no viable seeds were produced.

DNA blot analyses detected a single T-DNA insertion upstream of At5g64300 (AtRibA1) in rfd1 DNA. Exons 1–7 are indicated by red arrows, the mRNA sequence by a gray box, pWA5-derived T-DNA is inserted upstream of the AtRibA1 5’ end. The inserted sequence contains a 35S enhancer tetramer next to the right border (RB) sequence. The phosphinotricine resistance marker gene (pat) is controlled by a CaMV 35S promoter (35S prom.). Primers used in (F) are indicated by blue arrows. Since the schemes of AtRibA1 and the inserted T-DNA are scaled differently, sizes of both DNA stretches are given in parentheses. (G) Detection of T-DNA in individual etiolated non-fluorescent (lanes 1–4) or red fluorescent seedlings (5–7) of line rfd1. The Primer pair F1/R1 specifically amplified the wild-type allele (upper panel), whereas primer combination F1/LB1 detected AtRibA1-alleles containing integrated pWA5 T-DNA (lower panel). Arabidopsis wild-type and nontemplate controls are shown in lanes 8 and 9, respectively.
under normal light conditions confirmed the presence of a single T-DNA insertion. Arabidopsis genomic regions flanking the T-DNA insertion were identified by adapter ligation PCR methods. The right border of pWA5-derived T-DNA is inserted 307-bp upstream of the coding region of At5g64300, which encodes AtRibA1, the first enzyme of the plant riboflavin biosynthesis pathway. Arabidopsis RibA1 specifies a bifunctional GTP cyclohydrolase II/3, 4-dihydroxy-2-butanone 4-phosphate (DHBP) synthase that is able to complement Escherichia coli ribA and ribB mutant strains, respectively (12).

Amplification of the Arabidopsis genome region spanning the T-DNA insertion site from individual etiolated seedlings revealed a homozygous state of the T-DNA insertion in those seedlings that exhibited the red fluorescent phenotype (Figure 1G, lanes 5–7). Seedlings that did not over-accumulate Pchlde in the dark were either heterozygous or lacked the pWA5 T-DNA (Figure 1G). Analysis of light-grown seedlings (see Figure 1D) revealed, in line with the findings described for etiolated plants, that white individuals had no wild-type allele of AtRibA1 (data not shown). Hence, seedlings heterozygous for the rfd1 T-DNA insertion did not develop a visible phenotype, neither in the light nor under etiolated conditions (shown for nonfluorescent seedlings in Figure 1G). The term ‘rfd1 mutant’ therefore in the following refers to line rfd1 individuals that are homozygous for the pWA5 T-DNA insertion as indicated by the occurrence of the described phenotype(s).

The insertion upstream of the coding region of At5g64300 was shown to be responsible for the mutant phenotype of rfd1 by two different experimental approaches. First, addition of riboflavin suppressed the phenotype of At5g64300 was shown to be responsible for the mutant phenotype described in the following refers to line rfd1 individuals that are homozygous for the pWA5 T-DNA insertion as indicated by the occurrence of the described phenotype(s).

The insertion upstream of the coding region of At5g64300 was shown to be responsible for the mutant phenotype of rfd1 by two different experimental approaches. First, addition of riboflavin suppressed the phenotype of At5g64300 was shown to be responsible for the mutant phenotype described in the following refers to line rfd1 individuals that are homozygous for the pWA5 T-DNA insertion as indicated by the occurrence of the described phenotype(s).

Second, the introduction of an 5-kb genomic At5g64300 fragment spanning from F2 to R2 (see Figure 1E) using plasmid pRIB into rfd1 yielded plants homozygous for rfd1 but lacking the described phenotype. Heterozygous rfd1 individuals were transformed using pRIB and F1 transformants were tested for the presence of pWA5 by PCR. F2 offspring was analyzed for the rfd1 phenotype and the presence of integrated pRIB and pWA5 (i.e. rfd1) T-DNA. In total, 29 of 39 (i.e. 74%) analyzed F2 individuals contained integrated pRIB T-DNA. Seven seedlings (18%) displayed the rfd1 mutant phenotype and were shown by PCR to be homozygous for rfd1 but lacking the pRIB fragment. Five of the 39 analyzed seedlings (13%) were homozygous for rfd1 but contained the pRIB insert. All of these five individuals lacked the rfd1 mutant phenotype. The introduction of a wild-type AtRibA1 copy was hence able to complement the rfd1 mutant.

Transcript analyses
Surprisingly, transcript studies by semi-quantitative (sq) RT-PCR revealed a strong increase in transcription of AtRibA1 in rfd1 mutants in comparison to wild-type seedlings. Moreover, enhanced transcript accumulation was observed not only in homozygous, but also in heterozygous rfd1 individuals displaying no visible phenotype (Figure 2A). To investigate the structure of the over-accumulating transcripts, Northern blot analyses were performed. Due to limiting amounts of etiolated seedling material, heterozygous rfd1 plants grown on soil were used. The RNA blots revealed a drastically elongated AtRibA1 transcript of about 5.5-kb size in rfd1 plants (Figure 2B). In contrast, wild-type RibA1 mRNA has a size of 2.285 kb (11).

To characterize the 5.5-kb transcript, three further DNA fragments were used in Northern blot hybridization experiments. Probing with fragments of neighboring genomic regions (probes I/III in Figure 2C and D) did not detect the elongated AtRibA1 transcript. Only a probe specific to the T-DNA right border region detected the 5.5-kb transcript demonstrating that it originated inside the T-DNA region (probe II in Figure 2C and D). According to the size of the transcript and the fact that its AtRibA1 cDNA part was demonstrated to be correctly spliced (data not shown), the 5.5-kb RNA is derived from the 35S promoter driving the pat gene, which initiates transcription at about 3.2-kb upstream of the pWA5 T-DNA right border (red arrow in Figure 2D). To analyze transcript initiation upstream of pat, we used a 5′ RACE strategy based on ligation of a RNA adapter to Arabidopsis total RNA treated with calf intestine phosphatase (CIP) and tobacco acid pyrophosphatase (TAP) (6). The incubation with CIP dephosphorylated the 5′ mRNA ends, which were not protected by a 7-methylguanosine cap. The cap itself was converted in the subsequent TAP treatment step to 5′ monophosphate, which was then ligated to an RNA adapter. Reverse transcription with pat-specific primers was followed by primary RACE amplification (Figure 2E, upper panel). Primary PCR amplifications of homo- and heterozygous rfd1 plants were compared with an alternative pWA5 T-DNA containing Arabidopsis mutant (rfd4 in Figure 2E), which does not show enhanced pat gene transcription in northern blot analyses (data not shown). It was confirmed that transcripts initiated upstream of pat overaccumulated in rfd1. Secondary RACE PCR amplifications illustrate that the enhanced transcription initiation originates at or in close proximity to the pat TSS controlled by the 35S promoter in rfd4 (Figure 2E, lower panel). Hence, accumulation of the 5.5-kb T-DNA:AtRibA1 fusion transcript is the result of an enhanced 35S promoter activity in rfd1.

To understand the 5′ structure of the Arabidopsis RibA1 gene and, consequently, the misregulation in rfd1 we inspected transcription initiation sites in wild-type plants. Available EST sequences including the 5′ region of AtRibA1 extended to nucleotide +55 (Acc. Nr. CB254432). Herz et al. (11) described 5′ RACE analyses detecting capped transcripts starting at nt. –284. We performed 5′ RACE reactions as described for pat (see above) using now RibA1-specific primers in reverse transcription and amplification. We identified two major transcript start sites in A. thaliana RNA (Figure 3A, Wt). The majority of transcripts in wild-type plants was initiated at nt. –210 (Transcription Start Site 2 = TSS2) while a minor amount of 5′ RACE amplicons reached to nucleotides
–281/–285 (TSS1). The latter positions agreed well with the initiation at nt. –284 described by Herz et al. (12).

Publicly available T-DNA mutant collections harbor several lines with insertions in At5g64300. There were no candidates with T-DNA integrated in the coding region, but two insertions are localized in the 5' UTR. These insertion mutants with indicated T-DNA integrations at nt. –169 (SALK line 094736) and nt. –154 (SALK line 036891), respectively, proved to be useful, since they are situated downstream of the above characterized major TSS of AtRibA1. To characterize the T-DNA integration sites for both SALK lines in detail, the Arabidopsis genomic DNA neighboring the T-DNA border sequences on both sides were amplified and sequenced (Figure 3C). Mutants homozygous for the AtRibA1-specific insertions were produced by selfing and analyzed for the phenotype described for rfdl. While the described insertion sites were confirmed for both lines, neither etiolated nor light-grown mutant seedlings showed visible aberrations of homozygous SALK mutants in comparison to wild-type plants. Northern blots revealed in homozygous individuals of SALK lines 094736 and 036891 an accumulation of AtRibA1 mRNA to significantly lower, but still detectable levels (Figure 3B).

Mapping of AtRibA1 TSS in transgenic lines by 5' RACE

We analyzed mutants of both SALK lines by 5' RACE and mapped transcription initiation sites that were situated at nt. –130 (094736) and nt. –154 (SALK line 036891), respectively, proved to be useful, since they are situated downstream of the above characterized major TSS of AtRibA1. To characterize the T-DNA integration sites for both SALK lines in detail, the Arabidopsis genomic DNA neighboring the T-DNA border sequences on both sides were amplified and sequenced (Figure 3C). Mutants homozygous for the AtRibA1-specific insertions were produced by selfing and analyzed for the phenotype described for rfdl. While the described insertion sites were confirmed for both lines, neither etiolated nor light-grown mutant seedlings showed visible aberrations of homozygous SALK mutants in comparison to wild-type plants. Northern blots revealed in homozygous individuals of SALK lines 094736 and 036891 an accumulation of AtRibA1 mRNA to significantly lower, but still detectable levels (Figure 3B).
transcription from downstream sites when disrupted by T-DNA integration events. Surprisingly, in line 036891, we amplified a minor fraction of 5′RACE products that showed initiation taking place even inside the T-DNA region (–154 + 14 nt of left border sequence of pROK2 T-DNA). Since the left border sequence of pROK2, the vector used to generate the SALK T-DNA lines (2), is not known to encode a promoter, transcription initiation in this T-DNA tagged line seems to be partly determined by promoter structures situated downstream of the actual transcription start site.

Hence, transcript analyses of SALK lines 036891 and 094736 proved that a T-DNA insertion in the 5′UTR of AtRibA1, i.e. downstream of TSS1 and TSS2, can strongly reduce transcript accumulation, but does not automatically abolish transcript initiation. Furthermore, these T-DNA insertions fail to cause a phenotype resembling that of rfd1.

According to the scanning model of ribosome action (13), the over-accumulating 5.5-kb RNA in rfd1 can be assumed to be untranslatable (see ‘Discussion’ section). We therefore inspected rfd1 for the presence of translatable transcripts initiated around the wild-type TSS. The detection by Northern blot technique was infeasible due to limitations in available red fluorescent seedling material. Therefore, etiolated rfd1 seedlings exhibiting the red fluorescent phenotype were analyzed by 5′RACE. Experiments were performed in parallel with 5′RACE.
RACE reactions described above for wild-type plants and SALK lines (Figure 3A). However, in contrast to the latter, no transcription initiation was detectable upstream of AtRibA1 in the homozygous rfd1 seedlings (Figure 3A, lane rfd1). On the contrary, heterozygous rfd1 plants (Figure 3A, het.) yielded RACE products of identical size as observed in wild type. This proves that only homozygous rfd1 individuals are deficient in AtRibA1 mRNA initiated at the wild-type TSS. Hence, a complete lack of properly initiated transcripts in homozygous mutants coincides with the appearance of the characteristic phenotype of rfd1 (see Figure 1A and D).

**DISCUSSION**

In contrast to the implications of an interruption of an ORF by a T-DNA integration event, insertions in 5' UTR regions often do not display a 'knockout' phenotype. In agreement with this common observation, we demonstrated by using two different SALK lines that an integration event in the 5' UTR region of AtRibA1 itself was not sufficient to completely abolish transcription (Figure 3A and B). However, the identified rfd1 mutant resembled a complete 'knockout'. The reversibility of the phenotype by complementation in combination with the presented 5' RACE analyses proved this assumption.

The integrated pWA5 T-DNA in rfd1 encodes a 35S-enhancer tetramer at the right border sequence neighboring AtRibA1 (Figure 1E). The enhancer did not reinforce the transcription from the AtRibA1 promoter in heterozygous rfd1 plants (Figure 2B and C) or homozygous rfd1 mutant seedlings (see Figure 3A, lane rfd1). Instead, a strong overexpression starting at the T-DNA internal 35S promoter directing pat expression was observed (Figure 2D and E).

The accumulating 5.5-kb transcript comprises 3.2 kb of T-DNA sequence, followed by the AtRibA1 mRNA. By complementation experiments we showed firstly that the phenotype observed in homozygous mutants is abolished by riboflavin (Figure 1C). Second, the introduction of a genomic fragment carrying AtRibA1 promoter in heterozygous rfd1 plants (Figure 2B and C) or homozygous rfd1 mutant seedlings (see Figure 3A, lane rfd1). Instead, a strong overexpression starting at the T-DNA internal 35S promoter directing pat expression was observed (Figure 2D and E).

The AtRibA1 open reading frame (ORF) is situated at the 3'end of the 5.5-kb transcript accumulating in rfd1. The elongated mRNA contains numerous ORFs starting with the PAT protein coding sequence conferring phosphinothricin resistance followed by at least four ORFs of 115 amino acid each on the 35S enhancer fragment. The AtRibA1 reading frame enconding the riboflavin biosynthetic protein is hence the last on a polycistrionic messenger. According to the scanning model of ribosome action (12) translation in eukaryotic mRNAs starts at the most 5' situated start codon. Re-initiation of translation at a downstream ORF is known to occur only following very short regulatory reading frames in 5'UTRs or when internal ribosome entry sites are present. The latter mechanism has so far only for viral polycistrionic RNAs convincingly been described (14). Hence, translation of AtRibA1 from the polycistrionic 5.5-kb RNA in rfd1 is not feasible.

We have used a microarray approach to further characterize the rfd1 mutant (Hedtke, unpublished data). Using this technique, transcripts containing AtRibA1 were shown to accumulate more than 8-fold in etiolated homozygous mutants compared to wild-type seedlings.

Since the 35S tetrameric enhancer sequence present in rfd1 has been shown to be able to enforce transcription in promoters over a distance as far as 11 kb (15) we have analyzed the annotated mRNAs residing in a region of 50 kb surrounding the T-DNA insertion site in rfd1. None of the transcripts encoded in this region was found to be significantly enhanced in etiolated rfd1 seedlings. This finding was supported by the fact that the rfd1 phenotype was only observed in homozygous individuals. Hence, the genetic characteristics of the mutant rule out that rfd1 is a simple activation phenotype.

A T-DNA insertion event itself in the promoter region of AtRibA1 does not cause a sufficient reduction of expression to explain the phenotype of rfd1. This was demonstrated using SALK mutant lines 036891 and 094736. In both cases, insertions reside downstream of the T-DNA integration site of rfd1 as well as of the major transcription start sites TSS1 and TSS2 of AtRibA1. However, the integrated T-DNAs in both lines still allow sufficient RNA synthesis starting at aberrant initiation sites (Figure 3A–C) to avoid phenotypic consequences.

The analysis of the AtRibA1 promoter in wild-type plants by 5' RACE analyses identified transcription start sites at positions –285 and –210. A genome-wide identification of Arabidopsis TSS tags containing cap signatures by Yamamoto and Obokata (16) revealed AtRibA1 initiation sites at positions –285 and –76. Although we could not identify the TSS at nt –76 described by Yamamoto and Obokata in our 5' RACE reactions, obviously several motifs present in the AtRibA1 upstream region have the potential to serve as alternative promoters leading to various TSS. This is illustrated by the alternative transcription start sites detected in the two analyzed SALK lines (see Figure 3C).

That transcription of AtRibA1 in the SALK lines can be explained by an introduction or generation of new promoter structures as a possible consequence of the insertion of pROK2 is unlikely for two reasons. First, in both cases transcription starts at a different distance from the inserted LB pROK2 sequence. Second, there are no published hints neither on pROK2 left border internal promoters nor on a frequent generation of random promoters due to pROK2 T-DNA insertion events.

Most recently, a mechanism designated transcriptional interference was described in S. cerevisiae. The induction of the intergenic transcript SRG1 is able to silence the downstream situated, overlapping SER3 gene (5). Further investigation revealed that the transcription of noncoding SRG1 is serine-dependent and thereby adds an additional level of regulation on the expression of the serine biosynthetic gene SER3 (17). The regulatory
mechanism of transcriptional interference hence represents an activation event that directly represses a (downstream situated) gene.

Our data on transcription of AtRibA1 in rfd1 strongly suggest a transcriptional interference mechanism to be responsible for the lack of transcript initiation at the gene's own promoter in homozygous mutant individuals. First, the investigation of the two different SALK mutants proved that AtRibA1 promoter structure is highly flexible and tolerates T-DNA insertion events that are situated even closer to the translational start than the integration present in rfd1. Hence, the physical interruption of the AtRibA1 upstream region by the insertion of pWA5 T-DNA is highly improbable to be responsible for the observed phenotype. Second, by complementing the mutant with a genomic fragment containing AtRibA1, we demonstrated that RibA1 silencing in homozygous rfd1 mutants is not caused by an induction of mRNA degradation, i.e. by co-suppression. This would be a trans effect that also would silence the introduced complementing genomic fragment, which is not observed.

Recent work on transcription in eukaryotic organisms revealed an unforeseen amount of RNA synthesis in non-protein-coding regions (18). Also, plant genomes produce numerous such RNAs, including large (i.e. >40 nt) transcripts that are designated as mRNA-like non-coding (mlnc) RNAs (19).

The mechanism shown here to silence the transcription of AtRibA1 in rfd1 mutants demonstrates that transcriptional interference can be used also in plant systems to negatively regulate gene expression. Therefore, among the noncoding RNAs described in Arabidopsis so far a number of transcriptional interferers might be expected.

ACKNOWLEDGEMENTS

We thank Thomas Altmann (Gatersleben) for the Arabidopsis insertion mutant population which was the basis of the performed mutant screen.

FUNDING

The Deutsche Forschungsgemeinschaft in subproject B9 of the collaborative research center SFB 429. Funding for open access charge: Deutsche Forschungsgemeinschaft (DFG).

Conflict of interest statement. None declared.

REFERENCES

1. Bouché, N. and Bouché, D. (2001) Arabidopsis gene knockout: phenotypes wanted. Curr. Opin. Plant Biol., 4, 111–117.
2. Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R. et al. (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science, 301, 653–657.
3. Weigel, D., Ahn, J.H., Blazquez, M.A., Borevitz, J.O., Christensen, S.K., Fankhauser, C., Ferrandiz, C., Kudla, J., Malancharuvil, E.J., Neff, M.M. et al. (2000) Activation tagging in Arabidopsis. Plant Physiol., 122, 1003–1013.
4. Springer, P.S. (2000) Gene traps: tools for plant development and genomics. Plant Cell, 12, 1007–1020.
5. Martens, J.A., Laprade, L. and Winston, F. (2004) Intergenic transcription is required to repress the Saccharomyces cerevisiae SERS gene. Nature, 429, 571–574.
6. Kühn, K., Weihe, A. and Börner, T. (2005) Multiple promoters are a common feature of mitochondrial genes in Arabidopsis. Nucleic Acids Res., 33, 337–346.
7. L.J. and Chory, J. (1998) Preparation of DNA from Arabidopsis. Methods Mol. Biol., 82, 55–60.
8. Strizhov, N., Li, Y., Rosso, M.G., Viehoever, P., Dekker, K.A. and Weisshaar, B. (2003) High-throughput generation of sequence indexes from T-DNA mutagenized Arabidopsis thaliana lines. Biotechniques, 35, 1164–1168.
9. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1998) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
10. Beale, S.I. (1990) Biosynthesis of the tetrapyrrole pigment precursor, d-aminolevulinic acid, from glutamate. Plant Physiol., 93, 1273–1279.
11. Meskauskiene, R., Nuter, M., Goslings, D., Kessler, F., op den Camp, R. and Apel, K. (2001) FLU: a negative regulator of chlorophyll biosynthesis in Arabidopsis thaliana. Proc. Natl Acad. Sci. USA, 98, 12826–12831.
12. Herz, S., Eberhardt, S. and Bacher, A. (2000) Biosynthesis of riboflavin in plants. The ribA gene of Arabidopsis thaliana specifies a bifunctional GTP cyclohydrolase II/3,4-dihydroxy-2-butanoate 4-phosphate synthase. Phytochemistry, 53, 723–731.
13. Kozak, M. (2002) Pushing the limits of the scanning mechanism for initiation of translation. Gene, 299, 1–34.
14. Kozak, M. (2005) A second look at cellular mRNA sequences said to function as internal ribosome entry sites. Nucleic Acids Res., 33, 6593–6602.
15. Kim, J.I., Stark, J.R., Jin, J.B., Li, P., Jeong, J.C., Baek, D., Lee, S.Y., Blakeslee, J.J., Murphy, A.S., Bohnert, H.J. et al. (2007) yucca6, a dominant mutation in Arabidopsis, affects auxin accumulation and auxin-related phenotypes. Plant Physiol., 145, 722–735.
16. Yamamoto, Y.Y. and Obokata, J. (2008) ppdb: a plant promoter database. Nucleic Acids Res., 36 (Database issue), D977–D981.
17. Martens, J.A., Wu, P.Y. and Winston, F. (2005) Regulation of an intergenic transcript controls adjacent gene transcription in Saccharomyces cerevisiae. Genes Dev., 19, 2695–2704.
18. Hüttelhofer, A., Schattner, P. and Polacek, N. (2005) Non-coding RNAs: Hope or hype? Trends Genet., 21, 289–297.
19. Rymarquis, L.A., Kastenmayer, J.P., Hüttelhofer, A.G. and Green, P.J. (2008) Diamonds in the rough: mRNA-like non-coding RNAs. Trends Plant Sci., 13, 329–334.