Endogenous Targets of Transcriptional Gene Silencing in Arabidopsis

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Transcriptional gene silencing (TGS) frequently inactivates foreign genes integrated into plant genomes but very likely also suppresses an unknown subset of chromosomal information. Accordingly, RNA analysis of mutants impaired in silencing should uncover endogenous targets of this epigenetic regulation. We compared transcripts from wild-type Arabidopsis carrying a silent transgene with RNA from an isogenic transgene-expressing TGS mutant. Two cDNA clones were identified representing endogenous RNA expressed only in the mutant. The synthesis of these RNAs was found to be released in several mutants affected in TGS, implying that TGS in general and not a particular mutation controls the transcriptional activity of their templates. Detailed analysis revealed that the two clones are part of longer transcripts termed TSI (for transcriptionally silent information). Two major classes of related TSI transcripts were found in a mutant cDNA library. They are synthesized from repeats present in heterochromatic pericentromeric regions of Arabidopsis chromosomes. These repeats share sequence homology with the 3' terminal part of the putative retrotransposon Athila. However, the transcriptional activation does not include the transposon itself and does not promote its movement. There is no evidence for a general release of silencing from retroelements. Thus, foreign genes in plants encounter the epigenetic control normally directed, at least in part, toward a subset of pericentromeric repeats.

INTRODUCTION

An appropriate balance between activation and repression of genetic information is intrinsic to any living cell. Tight control of gene expression is necessary for adaptation to environmental factors, regulation of physiological parameters, and development of specialized cell types within a multicellular organism. Differentiation, in particular, is the result of mitotically heritable changes in gene expression, where the acquired states of gene activity reach a certain epigenetic stability. This stability arises by strict control of gene activators, by regulation of transcript stability, by altering the transcriptional availability of the genetic information itself, or by some combination of these. Alteration of the transcriptional availability of genetic information may be based on stable epigenetic silencing of selected genetic loci. This type of silencing is also evident from unexpected repression of transgene activities in various experimental systems (reviewed in Depicker and Van Montagu, 1997; Stam et al., 1997; Henikoff, 1998; Vaucheret et al., 1998; Grant, 1999).

The silencing of transgenic loci has received particular attention in plants, because it reduces the reliability of transgenic approaches in biotechnology. Complex inserts containing rearranged multiple copies of a foreign DNA are particularly prone to gene silencing. Loss of transgene expression may occur by two different mechanisms. The first precludes transcription (transcriptional gene silencing, or TGS); the second targets selected transcripts for rapid degradation (post-transcriptional gene silencing, or PTGS). The triggers of the two processes appear similar, because the onset of both types of silencing correlates well with redundancy of genetic information: DNA repeats in the case of TGS (reviewed in Meyer, 1996; Henikoff, 1998) and internal RNA repeats or overproduction in PTGS (reviewed in Depicker and Van Montagu, 1997; Grant, 1999). PTGS events are not meiotically transmitted and need to be reestablished in each sexual generation. PTGS is not necessarily coupled with a modification of the DNA template, but increased amounts of DNA methylation within the protein-coding region of silenced genes have been observed (reviewed in Depicker and Van Montagu, 1997). In contrast, TGS is meiotically heritable and correlates well with DNA template modification, as manifested by hypermethylation of the promoters of silenced genes (reviewed in Finnegan et al., 1998), by local changes in chromatin structure (reviewed in Gregory and Hoerz, 1998), or both.
The majority of silencing studies with various plant systems have explored transgenes as indicators of the silencing process. Few examples of gene silencing do not involve transgenic loci. A small gene family in Arabidopsis encoding phosphoribosylanthranilate isomerase (PAI), an enzyme in the tryptophan biosynthetic pathway, is subject to transcriptional regulation of its activity (Bender and Fink, 1995). The PAI gene family in the ecotype Wassilewskija consists of four gene copies distributed between three chromosomal locations. In a mutant with two linked copies deleted, the low transcription level of the remaining two ectopic genes leads to the accumulation of a UV-fluorescent precursor of tryptophan. Frequent spontaneous activation of these “inefficient” genes, seen as nonfluorescent revertants, occurs at 1 to 5% per generation.

A further example of TGS of an endogenous gene was discovered during a search for novel mutant alleles of the SUPERMAN gene (Jacobsen and Meyerowitz, 1997). Several new alleles turned out to be the result of epigenetic silencing of the wild-type gene. A similar phenomenon was noted in nature, where an epimutation of the Lcyc gene altered flower morphology (Cubas et al., 1999). These examples of the effects of TGS on endogenous loci indicate its importance in the regulation of selected genes; however, the criteria for TGS target susceptibility are still poorly understood, and the natural targets of transcriptional silencing in a normal, wild-type plant have not been discovered. Although some have postulated that TGS in plants has a defense function against invasive DNA such as transposable elements (Matzke et al., 1996; Martienssen, 1998; Matzke and Matzke, 1998), the experimental data relating to this hypothesis are limited (Hiroyaka et al., 2000).

To determine the natural targets of transcriptional silencing and thus explore the role of TGS in plants, we examined a set of transcriptional silencing mutants isolated previously. Because these mutations affect the maintenance of TGS and are able to reactivate diverse transcriptionally silent transgenic loci (Finnegan et al., 1996; Furner et al., 1998; Mittelsten Scheid et al., 1998; Jeddeloh et al., 1999; Amedeo et al., 2000), it has been hypothesized that the mutations also reactivate endogenous chromosomal information that otherwise would be transcriptionally silent. Here, we report the discovery of genetic information under the control of TGS that is expressed in silencing mutants but silenced in wild-type plants.

RESULTS

Isolation of Transcriptionally Silent Information

Usually, strains that release TGS accumulate developmental abnormalities with time, probably because of the ectopic expression or suppression of various genes that influence fitness and development (Finnegan et al., 1996; Kakutani et al., 1996). To circumvent a laborious selection against such a background of activated subsidiary genes, we chose the mom1 mutant as experimental material. Its normal growth and development over several generations indicate that the mom1 mutation acts in a relatively specific manner, its reactivation being confined to only a subset of transcriptionally silent chromosomal information, which we aimed to define. Details of mom1 are described elsewhere (Amedeo et al., 2000).

Populations of mRNA from 2-week-old Arabidopsis seedlings of the parental line A carrying a silent hygromycin-resistance locus (Mittelsten Scheid et al., 1991) and its mutant derivative mom1 with the transgenic locus reactivated were compared by using the method of suppression subtractive hybridization (Diatchenko et al., 1996), a procedure that should enrich for RNA species present only in mom1 plants or present in greater abundance in mom1 plants. The subtracted cDNA library was screened by inverted RNA gel blot analysis (von Stein et al., 1997). Twelve of 500 cDNA clones selected initially showed increased abundance when hybridized to labeled mom1 cDNA. Direct RNA gel blot analysis comparing total RNA of the parental line A and the mutant line revealed a striking genotype-dependent differential expression for two of the 12 cDNA probes. These cDNA clones were named TSI-A and TSI-B (for transcriptionally silent information). Both were abundant in mom1 RNA but undetectable in line A (Figure 1A). Results of RNA run-on analysis in isolated nuclei showed that TSI was transcriptionally silenced in line A and wild-type plants (data not shown).

Expression of TSI in Other Genotypes Affecting Gene Silencing

To exclude the possibility that release of TSI silencing is restricted to mom1, several Arabidopsis mutants and transgenic strains affected in this epigenetic regulation were assayed. Impaired maintenance of transcriptional silencing was also assigned to eight som mutants (som1 to som8) (Mittelsten Scheid et al., 1998), based on the release of silencing from an inactive transgenic locus as in mom1. Indeed, all som mutants showed TSI expression (Figure 1B). The ddm1 mutation, identified to have decreased DNA methylation (Vongs et al., 1993), also releases TGS from different loci (J eddeloh et al., 1998; Mittelsten Scheid et al., 1998). som4, -5, -7, and -8 are alleles of ddm1 (J eddeloh et al., 1999), and TSI expression was also found in this mutant, as expected (Figure 1B). TSI was also derepressed in a transgenic line having a decreased amount of DNA methylation as a result of overexpression of DNA methyltransferase antisense mRNA (Finnegan et al., 1996) and in the ddm2 mutant (affected in the maintenance DNA methyltransferase gene; E. Richards, personal communication). The silencing mutants hog1 and sil1 (Furner et al., 1998) also expressed TSI (Figure 1B). Mutant sil2 showed no sign of TSI expression; however,
sil2 was identified on the basis of a rather subtle release-of-silencing phenotype (Furner et al., 1998).

TSI-A hybridization of RNA gel blots with total RNA prepared from 2-week-old seedlings of the mutant mom1 and parental line A.

interestingly, the polyadenylated RNA fraction contained only the 5000- and 2500-nucleotide transcripts that hybridized to both cDNA probes (TSI-A and TSI-B) (Figure 1A). The TSI expression pattern was heritable and persisted through several selfed generations of the mutants (data not shown). Comparison of patterns of TSI-A expression revealed minor genotype-specific differences in the stoichiometry of the different
RNA species (Figure 1B). However, it is conceivable that general features of TSI transcripts are similar in all TGS mutants currently available.

**Organization of Chromosomal TSI Templates**

To determine the source of TSI transcripts and the organization of their template(s), TSI-A and TSI-B were used as probes for DNA gel blot analysis. The blots revealed that multiple copies of TSI-A- and TSI-B-homologous sequences are present in the genome of Arabidopsis (Figure 2). The copy number of TSI-A, as assessed by reconstruction experiments, was ~200.

To examine the degree of evolutionary conservation of the TSI arrangements, the DNAs of five Arabidopsis ecotypes (Zürich, Columbia, Landsberg erecta, Wassilewskija, and C24) were compared by DNA gel blot analysis and by hybridization to a TSI-A probe terminated at the DraI site, which has two recognition sequences within TSI-A (Figure 2A). An important conservation of the TSI-A pattern among different ecotypes was observed, with two main DraI repeats of 4 and 1.3 kb. Some minor differences specific for a particular ecotype indicated a limited genetic polymorphism within TSI-A. Probing the same membrane with TSI-B revealed complex banding patterns with differences in each ecotype (Figure 2A), which might indicate a much lower degree of conservation for TSI-B. However, the differences in DNA gel blot patterns between TSI-A and TSI-B can also be explained if TSI-A is an internal part of a longer repeated element and TSI-B is located proximal to a flank between repeated elements and variable single-copy DNA regions.

TSI repeats were heavily methylated in wild-type Arabidopsis and line A (Figure 2B); however, they were less methylated in mutants with genome-wide decreased DNA methylation (Figure 2B and data not shown). Importantly, TSI sequences remained methylated in the mom1 mutant (Figure 2B), in spite of their expression. This is analogous to the maintenance of methylation at the reactivated transgene in the mom1 background (Amedeo et al., 2000).

TSI-A and TSI-B were hybridized to the CIC yeast artificial chromosome library covering four equivalents of the Arabidopsis genome (Creusot et al., 1995). Sixty-two CIC clones out of 1152 were positive with the TSI-A probe. Twenty-six of these also contained the pericentromeric 180-bp repeat (Martinez-Zapater et al., 1986; Maluszynka and Heslop-Harrison, 1991), seven contained 5S RNA genes known to be located in the vicinity of a centromere (Bevan et al., 1999), and 16 clones contained other markers that mapped close to centromeres. Only four positive clones mapped outside of centromeric regions. Mapping of TSI-B resulted in hybridization to all TSI-A-positive CIC clones, with an additional seven clones hybridizing to TSI-B only. Thus, both TSI repeats are concentrated in the pericentromeric regions of Arabidopsis chromosomes. This was confirmed by cytological analysis of pachytene chromosomes with fluorescent in

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**Figure 2.** DNA Gel Blot Analysis in Various Ecotypes and Mutants.

(A) Conserved hybridization pattern of TSI-A in various ecotypes. DNA gel blot analysis was performed with ~5 μg of DNA from various ecotypes (Zh, Zürich; Col, Columbia; Ler, Landsberg erecta; Ws, Wassilewskija; and C24) digested with DraI. The blot was hybridized to a TSI-A probe (left) flanking the DraI site (see map below the gel) and rehybridized to TSI-B (right). Differences in hybridization intensity are the result of loading differences. DNA fragment lengths are indicated in kilobases.

(B) TSI methylation states in different mutants. DNA gel blot analysis was performed with ~5 μg of DNA (Zh, Zürich; line A, parental line of som5 and mom1; som5 and mom1 silencing mutants) digested with the methylation-sensitive restriction enzyme HpaII (left) and the methylation-insensitive restriction enzyme DraI (right) as a control.
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situ hybridization (FISH). These chromosomes are highly extended and span on average 66 μm (Fransz et al., 1998). TSI-A hybridization signals were found in the pericentromeric heterochromatin of all chromosomes (Figures 3A to 3C). A similar position was found for TSI-B sequences. Close examination revealed their presence in the heterochromatic domains flanking the centromeric 180-bp tandem repeat region. These regions are known to contain a high density of dispersed repeated elements (Copenhaver et al., 1999).

Multicopy TSI Templates Are Transcribed Unidirectionally

To determine the polarity of TSI transcription, RNase T and RNase A/T protection assays were performed with two TSI-A probes of opposite polarity. TSI-A was chosen as a probe because it is present in all transcripts detected on RNA gel blots (Figure 1). There was no evidence for specific protection of any TSI antisense RNA (Figure 4), suggesting a unidirectional transcription of the TSI templates. RNase digestions created a complex pattern of TSI-A–protected bands (Figure 4), which implies that many related but different RNAs hybridized to the probe. Furthermore, some of the protected TSI-A fragments are clearly more abundant than others, pointing toward either structural conservation of particular regions of TSI-A within related RNAs or a greater abundance of certain transcript subspecies.

Characterization of Transcripts Encoding TSI-A and TSI-B

The cDNA clones TSI-A and TSI-B had sizes of 903 and 614 bp, respectively. From the sizes of TSI transcripts detected on the RNA gel blots (Figure 1), it was obvious that both TSI clones represent only partial cDNAs. Because only two transcripts (5000 and 2500 nucleotides) were detected in the polyadenylated RNA fraction, this RNA was used for 5’ and 3’ extension reactions that started from the TSI-A sequence (Figure 5A). Two clones each were analyzed at the level of nucleotide sequences. The 5’ extension yielded inserts of 2512 and 1997 bp that were 97% identical in nucleotide sequences. The two clones from the 3’ extension were 1682 and 1652 bp and had 94% identity. Interestingly, both 3’ extension clones of TSI-A contained a region of 568 bp closely related to TSI-B (77% identity). This explains the detection of similar RNA species on RNA gel blots with TSI-A and TSI-B as probes and suggests that TSI-A and TSI-B are part of the same polyadenylated transcript species (Figure 1). To test whether the 5’ extensions of TSI-A are indeed part of the TSI transcript, RNA gel blots were probed with a cDNA fragment close to the 5’ end of the extension (Figure 5A, probe TSI-C). Interestingly, this probe hybridized only to the largest transcript of

Figure 3. FISH Images of Pachytene Chromosomes of Landsberg erecta Hybridized to TSI-A and the 180-bp Repeat.

(A) 4’,6-Diamidino-2-phenylindole (DAPI)-stained chromosomes.
(B) Hybridization signals of TSI-A (green) and the 180-bp repeat (red).
(C) Merged DAPI and FISH images showing the TSI-A signals in the pericentromeric region of all chromosomes. Bar in (A) = 5 μm for (A) to (C).
The 5000 nucleotides, which is also present in the poly(A) fraction (not shown). Because this transcript class also hybridized to TSI-A and TSI-B, the 5000-nucleotide transcripts probably contain a particular order of all three sequence elements (TSI-C, TSI-A, and TSI-B) (Figure 5).

The results of the RNase protection assay and the marked sequence heterogeneity between the duplicates of the 5’ and 3’ extensions of TSI-A indicated that they are transcribed from different but related DNA sequences. To determine the composition of TSI transcripts, a cDNA library prepared from mom1 seedlings was screened with two probes (TSI-A or TSI-B) (Figure 5). Among $5 \times 10^5$ clones screened, 179 were positive with at least one probe. Importantly, 94 clones hybridized to both TSI-A and TSI-B, confirming the 3’ TSI-A extension data (Figure 5). Twenty random cDNAs averaging 1.7 kb in size were subjected to sequencing. The sequence comparison revealed two major transcripts, represented by seven and six cDNAs with identical sequences within the overlapping regions (Figure 6). The remaining cDNAs were unique and probably represent transcripts of lesser abundance. Interestingly, the cDNAs fell into two classes, each showing sequence identity $>85\%$ among its members but $<70\%$ identity between the classes (Figure 6).

A search within an Arabidopsis genomic sequence database uncovered a chromosomal DNA stretch (bacterial artificial chromosome [BAC] F7N22) identical to one of the abundant cDNAs (Figure 6). This sequence, annotated as Athila repeat, is located at the pericentromeric region of chromosome 5 and was recently used in comparative studies of various Athila-like repeats (Wright and Voytas, 1998). Athila was isolated in a search for sequences flanking the 180-bp repeat; it is classified as a retrotransposon based on the presence of long terminal repeats, a polyuridine tract, and a primer binding site for tRNA priming of the reverse transcriptase (Péllissier et al., 1995, 1996). However, the two open reading frames (ORF) of Athila (Figure 5B, ORF1 and ORF2) have no homology with proteins usually encoded by retroelements or any other known protein, and neither movement nor even transcriptional activity has ever been observed.

Both TSIs and all sequenced cDNA clones mapped to the 3’ terminal half of the Athila element (Figures 5A and 5B). All TSI regions homologous to the Athila ORF2 appear to be degenerated coding regions: the ORFs encoded by the two cDNAs derived from the 5’ extensions of TSI-A are interrupted by translational stop codons after 398 (clone a) and 83 (clone b) amino acids. The sequence corresponding to ORF2 present on the BAC F7N22 is modified by five deletions of 2 to 31 bp and five insertions of 3 to 10 bp (Figure 5B). This indicates significant deviation of these sequences from that of the putative retrotransposon.

The sequence information of BAC F7N22 was used to determine the position of the transcription start for the longest TSI transcript. First, reverse transcription–polymerase chain reaction (RT-PCR) amplifications with primers spanning the region of the putative start site as expected from the longest transcript size were performed (Figure 5B). Results indicated that transcripts start downstream of position 64,929. Accordingly, an antisense RNA probe for RNase A/T protection was produced spanning the 638 nucleotides between positions 64,929 and 65,567. Protected fragments of ~480 ± 10 nucleotides (Figure 7) allowed positioning of the TSI transcription start on BAC F7N22 to 65,087 ± 10 nucleotides. Because RNA preparations from ddm1, som7, and mom1 protect fragments of the same size (Figure 7), TSI transcripts start at similar sites in different mutant backgrounds.
Selectivity of TSI Activation

The TSI identified in the differential screen and the clones recovered from the cDNA library were homologous to only the 3' half of the Athila-like element, and the transcription start of the unidirectional transcripts was mapped to the middle of the putative retroelement. Therefore, we examined whether the 5' part of the Athila-like sequence present on BAC F7N22 (including the first ORF; Figure 5) was reactivated in TGS mutants. RNA gel blot analysis with a probe spanning a 1.6-kb region of ORF1, as well as RT-PCR analysis with primers from the same region, gave negative results (data not shown). This suggests that expression of TSI is restricted to the 3' half of Athila-like sequences. In addition, we failed to detect extrachromosomal DNA indicative of the replication of retroelements (Peleman et al., 1991), suggesting that TSI sequences are not part of a replicative transposon.

The two classes of isolated cDNAs shared only ~50% identity with Athila (Figure 6). To address directly the question of whether Athila is expressed, RT-PCR experiments were performed with Athila-specific primers in the TSI homologous region (Figure 5). However, the corresponding fragment could not be amplified from RNA of mom1 seedlings, suggesting that only a subset of Athila-like sequences but not the Athila element itself was reactivated in the mutant background.

The homology of TSI to the putative retrotransposon also raised the question of whether other endogenous retroelements are transcriptionally reactivated. Reverse transcriptase is the most highly conserved protein encoded by retroviruses and retrotransposons (Doolittle et al., 1989). The same degenerated primers in a conserved region of the reverse transcriptase gene that were applied to clone the Ta superfamily of Arabidopsis retrotransposons (Konieczny et al., 1991) were used to investigate whether members of the Ta family are transcribed in the mutant background. Although the expected 268-bp fragment was amplified from genomic DNA, no amplification was achieved in RT-PCR with mom1 RNA as template (data not shown). Thus, retrotransposons are not generally reactivated in the mom1 mutant.
The repetitive nature of TSI and its pericentromeric location led to the question of whether other pericentromeric repeats are expressed in TGS mutants. Athila sequences (Pélissier et al., 1995, 1996) are often flanked by copies of the 180-bp repeat (Martinez-Zapater et al., 1986), and transcripts could originate from read-through transcription. No transcripts homologous to these repeats were detectable by RNA gel blot analysis (data not shown). Thus, the release of silencing appears to affect selected templates only. What distinguishes these targets from other, apparently related repeats remains to be investigated.

**Fast-Growing Suspension Culture Cells Express TSI**

TSI expression in the wild-type background was not detected either in young seedlings (2 weeks old) or in different tissues of mature wild-type plants (roots, shoots, leaves, flowers, and siliques). The application of various stress treatments (increased salinity, UV-C irradiation, or pathogen infection) also did not activate TSI in wild-type plants (data not shown). Furthermore, TSI expression was not detected in freshly initiated callus cultures, and transcriptional suppression of TSI was stable even after several in vitro passages of the callus culture. The only exception so far is a fast-growing, long-term suspension culture derived from wild-type Arabidopsis (May and Leaver, 1993). These cells expressed TSI to approximately the same extent as the mutants (Figure 8), indicating the release of epigenetic TSI silencing under these conditions.

**DISCUSSION**

The epigenetic control of gene activity in plants can be exerted either in a sequence-specific manner, for example, by polycomb-type proteins (Goodrich et al., 1997), or in a broader way through regional changes in DNA methylation or chromatin properties (reviewed in Richards, 1997). TGS seems to reflect the latter, more global type of epigenetic control (Chen and Pikaard, 1997; Jeddeloh et al., 1998; Mittelsten Scheid et al., 1998). However, even a general system requires other, probably sequence-independent means to recognize its targets. A trait suspected to trigger silencing is genetic redundancy in the form of duplications and repetitive structures (reviewed in Meyer, 1996; Selker, 1997; Henikoff, 1998). Indeed, genetic modifier loci of transcriptional silencing have been identified by their action on a highly repetitive transgenic locus (Mittelsten Scheid et al., 1998), and some of them were shown to be allelic to the ddm1 mutation (Jeddeloh et al., 1999), which acts primarily and immediately on the methylation status of the abundant 180-bp centromeric repeat (Vongs et al., 1993). Although the activity of several low-copy genes also can be affected by disruption of genome-wide methylation patterns (Jacobsen and

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**Figure 6.** Relationships between TSI Transcripts Isolated from a mom1 cDNA Library and Their Potential Genomic Templates.

The comparison is based on a 400-bp sequence within the 3' non-coding region of TSI (indicated in Figure 5). The numbers indicate the number of cDNA clones with identical sequence. The bar represents 10 substitutions per 100 residues.

**Figure 7.** RNase A/T Protection Assay to Map the 5' Transcription Start.

Ten micrograms of total RNA of ddm1, som7, mom1, and yeast were hybridized to an antisense RNA probe spanning the region of the transcription start as determined by RT-PCR experiments. The protected fragment had a size of ~480 nucleotides in all mutants assayed. Molecular sizes are indicated (in nucleotides; nt) next to the gel.
it would be inappropriate to consider them as primary targets for methylation-mediated epigenetic control, given the stochastic nature and slow progress of silencing. Their altered activity is more likely to be a secondary effect of the accumulation of an epigenetic disorder in the mutant background (Finnegan et al., 1996; Kakutani et al., 1996; Jeddeloh et al., 1999). The primary endogenous targets of the transcriptional silencing system were not known previously, and there was no evidence for expression of the demethylated 180-bp repeats in the mutants (Vongs et al., 1993; Kakutani et al., 1999). The search for plant-specific TGS targets described in this article has now disclosed a class of repetitive elements as being more promising candidates.

The TSI present in the genome of wild-type plants was stimulated to produce RNA in a range of mutants that are affected in the maintenance of transcriptional silencing. Their activity is more likely to be a secondary effect of the accumulation of an epigenetic disorder in the mutant background (Finnegan et al., 1996; Kakutani et al., 1996; Jeddeloh et al., 1999). The primary endogenous targets of the transcriptional silencing system were not known previously, and there was no evidence for expression of the demethylated 180-bp repeats in the mutants (Vongs et al., 1993; Kakutani et al., 1999). The search for plant-specific TGS targets described in this article has now disclosed a class of repetitive elements as being more promising candidates.

The TSI present in the genome of wild-type plants was stimulated to produce RNA in a range of mutants that are affected in the maintenance of transcriptional silencing. An initial search for TSI, we isolated two independent cDNA clones representing RNA specifically expressed in silencing mutants. Because one would anticipate that there are several DNA templates suppressed by the silencing system in wild-type plants, we find it remarkable that the corresponding cDNAs are closely related to each other or are even parts of the same transcript. This may indicate that the TSI recovered here represents a tightly regulated template with no background expression in the wild type, which is thus an optimal substrate for suppression cloning. Alternatively, the TSI described could be a very efficiently transcribed template or templates in the absence of the silencing control. In any case, the expression of TSI seems to be a very specific marker of disturbed silencing.

The three main TSI transcripts of 5000, 2500, and 1250 nucleotides, originating from unidirectional transcription, contain an element isolated as TSI-A. It is not clear whether these represent separate transcriptional units regulated by different promoters or whether they are processing products of the longest transcript. The 5000- and 2500-nucleotide transcripts are polyadenylated, but the most abundant transcript of 1250 nucleotides is absent from the poly(A) fraction and thus possibly is retained in the nucleus. The most abundant transcripts have no protein-coding capacity, and only the longest mRNA includes sequences related to the ORF2 of the putative retrotransposon Athila. However, all TSI sequences analyzed so far contain only degenerated regions of ORF2. Therefore, TSI probably belongs to a class of non-coding RNA.

Sequence analysis of 20 randomly chosen TSI cDNA clones derived from mom1 RNA indicated overrepresentation of two transcripts and the presence of several less abundant but related RNAs. Because no two identical TSI repeats were found in the Arabidopsis genomic sequence available (covering >80% of the genome), probably various TSI templates are transcribed with differing efficiencies or perhaps selected TSI transcripts have increased stability.

No putative function for TSI was revealed by sequence comparison with protein- or RNA-coding sequences. The only similarity, limited to ~50%, was found with the 3’ half of the putative degenerated retrotransposon Athila (Pélissier et al., 1995, 1996; Wright and Voytas, 1998). The 5’ part of Athila directly adjacent to the TSI template region was not reactivated in the silencing mutant. Furthermore, not all Athila copies are reactivated in the mutant background, and no reactivation of other Arabidopsis retroelements, for example, the Ta superfamily (Konieczny et al., 1991), was detected. This suggests that TGS is not directed toward retrotransposons in general, although its targets may have originated from ancient transposition events. Recently, a high selectivity of TGS also in respect to transposon targets has been revealed (Hirochika et al., 2000).

So far, and based on the complete sequence of two Arabidopsis chromosomes (Lin et al., 1999; Mayer et al., 1999), Athila elements were found only in heterochromatic regions of chromosome 4 (Mayer et al., 1999; Fransz et al., 2000; McCombie et al., 2000). However, none of the TSI cDNAs could be aligned to the sequenced chromosomes without numerous mismatches. Therefore, the presence of TSI templates within chromosome 2 and 4 is still unclear, and the two most abundant cDNAs must be transcribed from the
remaining chromosomes. Indeed, the template for one of them was found on BAC F7N22 located on chromosome 5.

Repeats derived from degenerated retroelements have often been found in centromeric locations in fungi and plants (Cambareri et al., 1998; Presting et al., 1998; Copenhaver et al., 1999). Whether these repeats are important components of centromeric function, either by nature of their sequences or by their epigenetic modifications, remains a matter for discussion (reviewed in Csink and Henikoff, 1998; Copenhaver and Preuss, 1998). One of the features proposed as a prerequisite for centromere function is late replication of these heterochromatic regions (reviewed in Csink and Henikoff, 1998). If this was also true for Arabidopsis centromeres, undue loosening of suppressive chromatin in the pericentromeric region leading to TSI expression could cause disturbances in mitosis, which would result in severe phenotypes (Liu and Meinke, 1998). However, sil1 and mom1 mutant plants exhibit no abnormalities suggestive of mitotic disorders. It is possible that TSI is transcribed in spite of the presence of the suppressive chromatin environment, as was documented for the XIST locus transcribed from the inactive X chromosome (Gartler et al., 1999); thus, its activation does not interfere with pericentromeric functions. Importantly, not only strains affected in transcriptional silencing through alterations in genome-wide DNA methylation but also silencing mutants with unchanged methylation levels (sil1 and mom1) reactivate TSI. This indicates that release of epigenetic suppression from endogenous templates does not require the loss of methylation. Because the latter mutants exhibit no striking phenotypic alterations, release of TGS and TSI expression per se is not responsible for the multiple and variable morphological and developmental aberrations seen in methylation-affected mutants. Thus, at this point it is both evident and surprising that transcriptional reactivation of a subset of usually silent pericentromeric repeats, such as described here, has no immediate consequences. Therefore, their silencing may be important under specific, undefined conditions or on a longer time scale. Speculation about the biological role of TSI and the significance of its epigenetic suppression system would be premature. However, the epigenetic control of TSI resembles that of other noncoding RNAs and retroelements with better-defined functions, such as Xist involved in X chromosome inactivation (reviewed in Marahrens, 1999) or TART and HeT-A with telomeric function (reviewed in Pardue et al., 1997). Moreover, the TSI expression observed in suspension culture suggests that long-term, fast growth of dedifferentiated cells can select for an escape from epigenetic control. Such epigenetic imbalance could be the main origin of somaclonal variation (Phillips et al., 1994) and activation of transposable elements (Bretell and Dennis, 1991; Hirochika, 1993; Hirochika et al., 1996) during prolonged plant tissue culture.

The recognition signals designating a particular set of endogenous repeats or transgenes for silencing are still unknown. The affected sequences described here probably represent only the tip of an iceberg, and the discovery of further TSI elements should help resolve the role and mechanism of transcriptional silencing in plants. At present, the perfect correlation of TSI expression in all mutants known to affect transcriptional transgene silencing promotes these mutants to the status of powerful icebreakers. Results of the ongoing characterization of the mutated silencer components will help define their interaction with their targets. Furthermore, TSI itself provides a potent tool for uncovering novel genes involved in TGS, either in known mutants or in tagged mutant populations.

### METHODS

#### Plant Material

Arabidopsis thaliana seeds were surface-sterilized twice with calcium hypochlorite (5% with 0.1% Tween 80) for 5 min and washed with sterile double-distilled water. They were then plated in aseptic conditions on germination medium (Masson and Paszkowski, 1992) solidified with 0.8% agar. Seedlings were grown in a phytotron with cycles of 16 hr of light at 21°C and 8 hr of darkness at 16°C.

#### Differential mRNA Screening and Cloning Procedures

Total RNA was isolated according to Goodall et al. (1990) from 2 g (fresh weight) of 2-week-old seedlings from the mutant mom1 (Amedeo et al., 2000) and the parental line A (Mittelsten Scheid et al., 1991). Polyadenylated RNA was prepared by using Dynabeads Oligo (dT)25 (Dynal, Oslo). Suppression subtractive hybridization (Diatchenko et al., 1996) was performed with 2 µg of polyA RNA, using the PCR (polymerase chain reaction)-Select cDNA subtraction kit (Clontech, Palo Alto, CA) according to the supplier’s instructions. The mutant line was used as tester and the parental line A as driver cDNA population. The subtracted mom1 library was cloned into vector pCR2.1 (Invitrogen, Groningen). For the screening procedure, 500 individual bacterial cultures were grown according to the manual of the PCR-Select differential screening kit (Clontech). To reduce the number of false-positive clones, the library was also screened as described by von Stein et al. (1997).

5’ and 3’ extension reactions were performed with the Marathon cDNA amplification kit (Clontech) according to the manufacturer’s protocol. The sequence-specific primers (see Figure 2) were 5’TGGTTCACCAGATAAGCTAGTGCCCCTC-3’ (TA-F1) and 5’-CTTCAAGACTGGATAGGACTAGGCG-3’ (nested primer TA-F2) for the 3’-extension reaction, and 5’-CGCCCCACCTAGTGCTATCAGTGTCAGAGG-3’ (TA-R1) and 5’-CGCATCAAACAACTAACAACGGGACAC3’ (nested primer TA-R2) for the 5’-extension reaction. PCR products were cloned into vector pCR2.1 (Invitrogen). Individual bacterial cultures were grown and subjected to colony PCR as described in the manual of the PCR-Select differential screening kit (Clontech), with the primer combinations used to create the extension reactions. To screen for positive TSI-A extension clones, the PCR products were blotted and hybridized to TSI-A. All PCR reactions used for cloning procedures were performed with a polymerase mix with proof-reading capacity (Advantage cDNA PCR kit; Clontech).
RNA and DNA Gel Blot Analysis and Library Screens

Total RNA was isolated either as described by Goodall et al. (1990) or by the RNeasy plant mini kit (Qiagen, Hilden) according to the supplier’s instructions. For RNA gel blot analysis, the RNA was electrophoretically separated after denaturation by glyoxal in a 1.5% agarose gel in phosphate buffer, pH 7.0, and blotted to nylon membranes (Hybond N; Amersham) by using standard protocols (Sambrook et al., 1989). The molecular weight marker I (Roche Diagnostics, Rotkreuz) was used as a size standard. For DNA gel blot analysis, genomic DNA was isolated according to Dellaporta et al. (1983) and separated electrophoretically after endonucleolytic digestion. DNA fragments were also transferred to nylon membranes (Hybond N) by standard procedures (Sambrook et al., 1989). Hybridization and washing of all RNA and DNA gel blots and the filters with the yeast artificial chromosome library was performed according to Church and Gilbert (1984). Probes were labeled with α-32P-dATP by random primer DNA polymerization (Feinberg and Vogelstein, 1983). Blots were exposed to x-ray-sensitive films (Kodak X-OMAT AR).

For the mom1 cDNA library, polyadenylated RNA was prepared from 2-week-old mutant seedlings as described above. The cDNA library was prepared by using the Uni-Zap XR library construction kit (Stratagene) according to the manufacturer’s instructions. Long cDNAs were selected by using size fractionation columns from Gibco BRL.

RNase Protection Assays

The RNase protection assays were performed according to Goodall et al. (1990) with minor modifications. To assay the direction of TSI transcription, the pCR2.1 plasmid with the TSI-A insert was cut by EcoRI, creating a fragment of 781 bp, which was ligated into the pGEM-7Zf(-) vector (Promega). To map the 5’ transcription start, the probe was generated by amplifying by the bacterial artificial chromosome (BAC) F7N22 region between positions 64,929 and 65,567 and inserting the product into the pGEM-7Zf(+)+ vector (Promega). The labeled probes were synthesized by in vitro transcription of the linearized plasmid in the presence of α-32P-UTP by using T7 polymerase (Promega) or Sp6 polymerase (Roche Diagnostics) and purified by electrophoresis (Goodall et al., 1990). Hybridization was performed at 52°C. Single-stranded RNA was cleaved by either 4 μg of RNase A and 0.6 units of RNase T1 (RNase A/T assay) or by 20 units of RNase T1 (RNase T assay) for 40 min at 30°C. Protected fragments were separated on a denaturing 6% polyacrylamide gel. The dried gels were recorded on a PhosphorImager screen (Amersham Pharmacia Biotech, Uppsala).

Reverse transcription–PCR and PCR Reactions

Reverse transcription (RT) was performed with 1 μg of total RNA from mom1 in the presence of 1 mM dNTPs, 20 units of RNasin (Promega), AM RTase buffer (Roche Diagnostics), and 25 units of AM reverse transcriptase (Roche Diagnostics) at 37°C for 1 hr, followed by heat inactivation of the reaction mixture. The template for PCR was 100 ng of genomic DNA, 100 ng of cDNA, or 50 ng of reverse-transcribed RNA primed by gene-specific antisense primers. PCR was started with 3-min denaturation at 94°C, followed by 30 cycles (denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec, and elongation at 72°C for 30 sec) in the presence of 0.2 mM dNTPs, 0.4 μM forward and reverse primers in Taq DNA polymerase buffer (Roche Diagnostics), and 0.25 units of Taq DNA polymerase (Roche Diagnostics). The gene-specific primers for Athila (X81801) were derived from positions 8156 to 8184 (GS-F) and 8430 to 8458 (GS-R).

The TA-F1 and TA-R1 primers were used as a positive control for all RT-PCR reactions. The reverse transcriptase region of the pol gene of Arabidopsis Tyl/copia-like retrotransposon family was amplified as described by Konieczny et al. (1991) with minor modifications.

Stress Application (Salinity, UV-C, or Pathogen)

Induction of TSI by UV-C irradiation was tested on RNA gel blots with RNA samples from 1-week-old seedlings subjected to UV-C treatment of 1 or 5 kJ/m2 and collected at several time points within 1 hr (Revenkova et al., 1999). The effect of osmotic stress was tested on RNA gel blots with RNA from 1-week-old seedlings that were transferred for 24 hr to medium with NaCl concentrations of 0, 0.04, 0.08, or 0.12 M (Albinsky et al., 1999). To test TSI expression in conditions of pathogen stress, RNA samples from 3-week-old seedlings either mock-treated or infected with Peronospora (provided by R. Reist, Novartis Crop Protection, Basel) were analyzed by RNA gel blot analysis. To verify the appropriate pathogen response, induction of PR1 expression was monitored by reprobing the membrane with a PR1 probe (provided by R. Reist).

DNA Sequencing

Sequencing reactions were performed with Dye Terminators (conventional rhodamine terminators or dRhodamine terminators) from PE Applied Biosystems (Foster City, CA) using a Perkin-Elmer GeneAmp PCR system model 2400, 9600, or 9700 thermocycler and analyzed with an ABI PRISM 377 DNA sequencer.

Cytogenetic Analysis of TSI-A on Pachytene Chromosomes

Flower buds from wild-type Landsberg erecta were fixed in ethanol/acetic acid (3:1 [v/v]), washed in water, and incubated for 2 hr at 37°C with a combination of 0.3% (w/v) pectolyase (Sigma), 0.3% (w/v) cytochelicase (BioSepra, Idstein), and 0.3% (w/v) cellulase (Sigma) in 10 mM sodium citrate, pH 4.5, to remove the cell wall (Ross et al., 1996). The pachytene chromosomes were spread as described by Fransz et al. (1998). Pachytene chromosome preparations were baked at 60°C for 30 min. After incubating with RNase (10 μg/mL in 2 × SSC [1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate], the slides were rinsed twice in 2 × SSC buffer for 5 min and twice in PBS (10 mM sodium phosphate, pH 7.0, and 143 mM NaCl) for 5 min. The preparations were then fixed in 1% (w/v) paraformaldehyde in PBS for 10 min, rinsed twice in PBS for 5 min, dehydrated through an ethanol series (70, 90, and 100%; each 2 min), and air-dried.

Plasmid DNA with TSI-A or TSI-B was labeled with biotin-UTP or digoxigenin-UTP using the High Primed labeling kit of Boehringer Mannheim. The hybridization mix contained ~100 ng of labeled DNA in 50% formamide, 2 × SSC, 50 mM sodium phosphate, pH 7.0, and 10% dextran sulfate. Twenty microliters of hybridization mix was added to each slide. After denaturation at 80°C for 2 min, the slides were incubated at 37°C for 18 hr. Posthybridization washes were performed in 50% formamide in 2 × SSC, pH 7.0, three times for 5 min at 42°C followed by 2 × SSC at room temperature three times for
5 min. Immunodetection of the hybridization signals was performed according to Fransz et al. (1998). The fluorescence patterns were examined with a Zeiss (Jena) Axiosplan, using filter blocks for 4’,6-diamidino-2-phenylindole and fluorescein isothiocyanate. Selected images were recorded by conventional and charge-coupled device cameras. The digital images were processed to enhance brightness and contrast by using Adobe Photoshop (Adobe Systems, USA) computer software.

Database Searches

Sequence analysis was performed with the GCG software (Wisconsin Package Version 10.0; Genetics Computer Group, Madison, WI). For homology searches, GenEMBL, the Arabidopsis database (http://genome-www.stanford.edu/), the Kazusa Arabidopsis opening site (http://zearth.kazusa.or.jp/arabi/), and SwissProt (http://www.expasy.ch/sprot/) were used. Peptide sequences were analyzed by GeneQuiz (http://columba.ebi.ac.uk:8765/gqsrv/submit) or Expasy (http://www.expasy.ch/).

ACKNOWLEDGMENTS

We owe special thanks to Yoshiki Habu for his contribution to the manuscript. We thank Barbara Hohn, Thomas Hohn, Witold Filipowicz, Patrick King, Danielle Conroy, and Diana Pauli for helpful comments on the manuscript. We also thank Eric Richards, Ian Furner, Jean Finnegan, Roland Reist, Doris Albinsky, and Ekaterina Revenkova for providing biological material. Further, we acknowledge Herbert Angliker, Finnegan, Roland Reist, Doris Albinsky, and Ekaterina Revenkova for providing biological material. Further, we acknowledge Herbert Angliker, Maciej Pietrzak, and Peter Müller for sequencing and primer service. This work was supported by the Swiss Federal Office for Education and Science (BBW Grant No. 96.0250-1) and the European Union (EU Grant No. BIO4CT960253).

Received January 24, 2000; accepted May 18, 2000.

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Endogenous Targets of Transcriptional Gene Silencing in Arabidopsis
Andrea Steimer, Paolo Amedeo, Karin Afsar, Paul Fransz, Ortrun Mittelsten Scheid and Jerzy Paszkowski
*Plant Cell* 2000;12;1165-1178
DOI 10.1105/tpc.12.7.1165

This information is current as of August 14, 2017

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