

TECHNICAL ADVANCE

Transgene-induced RNA interference: a strategy for overcoming gene redundancy in polyploids to generate loss-of-function mutations

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Summary

Gene redundancy in polyploid species complicates genetic analyses by making the generation of recessive, loss-of-function alleles impractical. We show that this problem can be circumvented using RNA interference (RNAi) to achieve dominant loss of function of targeted genes. *Arabidopsis suecica* is an allotetraploid (amphidiploid) hybrid of *A. thaliana* and *A. arenosa*. We demonstrate that *A. suecica* can be genetically transformed using the floral dip method for *Agrobacterium*-mediated transformation. Transgenes segregate as in a diploid, indicating that chromosome pairing occurs exclusively (or almost so) among homologs and not among homeologs. Expressing a double-stranded (ds) RNA corresponding to the *A. thaliana* gene, decrease in DNA methylation 1 (*DDM1*) caused the elimination of *DDM1* mRNAs and the loss of methylation at both *A. thaliana*- and *A. arenosa*-derived centromere repeats. These results indicate that a single RNAi-inducing transgene can dominantly repress multiple orthologs.

Keywords: RNAi, DNA methylation, *DDM1*, *Arabidopsis suecica*, allopolyploid.

Introduction

Polyploidy has played a prominent role in the evolution of plants species. In fact, it is estimated that at least 70% of all angiosperms and 95% of all ferns underwent at least one episode of polyploidization in their evolutionary history (Comai, 2000; Leitch and Bennett, 1997; Masterson, 1994; Soltis and Soltis, 2000; Wendel, 2000). Numerous crop plants have long been recognized as polyploids; these include bread wheat, pasta wheat, coffee, potato, sugarcane, canola, and cotton. More recently, genome and expressed sequence tag (EST)-sequencing projects have revealed that ancient genome duplication events also occurred in maize and *Arabidopsis thaliana* (Gaut *et al.*, 2000; The-Arabidopsis-Genome-Initiative, 2000).

Polyploids can arise via duplications of a single genome (autopolyploidy) or by combining the genomes of two or more progenitors (allopolyploidy). The success of plant polyploids in nature is thought to be as a result of ecologic or population fitness advantages (Stebbins, 1971). One possibility is that the pairing of homologous

chromosomes (those derived from the same progenitor), but not homeologous chromosomes (equivalent chromosomes derived from different progenitors), in allopolyploids ensures the transmission of each set of chromosomes to successive generations. The resulting 'forced heterozygosity' might confer permanent hybrid vigor in an allopolyploid. Another idea is that the presence of multiple copies of each gene buffers against the deleterious effects of mutations while allowing for differences in the activity of encoded proteins to be exploited, thus allowing hybrids to thrive in a broader range of habitats than their progenitors do (Comai, 2000; Pikaard, 2001; Soltis and Soltis, 2000; Wendel, 2000).

Gene redundancy may have advantages in nature, but it makes polyploids poor choices for genetic analyses because of the difficulty of obtaining loss-of-function alleles. Nonetheless, a number of interesting genetic and epigenetic phenomena occur in polyploids, including gene loss and chromosome re-arrangements, changes in DNA

methylation, and changes in gene expression (Chen and Pikaard, 1997a,b; Comai, 2000; Comai *et al.*, 2000; Feldman *et al.*, 1997; Kashkush *et al.*, 2002; Lee and Chen, 2001; Matzke *et al.*, 1999; Mittelsten Scheid *et al.*, 1996; Osborn *et al.*, 2003; Ozkan *et al.*, 2001; Pikaard, 2001; Shaked *et al.*, 2001; Song *et al.*, 1995). An emerging model system for studies of polyploidy is *A. suecica* (Chen *et al.*, 1998; Comai *et al.*, 2000; Lee and Chen, 2001; Madlung *et al.*, 2002), an allotetraploid whose genome is composed of diploid chromosome complements derived from *A. thaliana* and *A. arenosa* (formerly known as *Cardaminopsis arenosa*; Al-Shehbaz and O'Kane, 2002; O'Kane, 1997; O'Kane *et al.*, 1995). In this report, we show that *A. suecica* can be genetically transformed using the floral dip method commonly used for *Agrobacterium tumefaciens*-mediated transformation of *A. thaliana* (Bechtold *et al.*, 1993; Clough and Bent, 1998). As a test case for the use of RNA interference (RNAi; Hannon, 2002; Matzke *et al.*, 2001; McManus and Sharp, 2002; Waterhouse and Helliwell, 2003) to knock down the activity of orthologous genes inherited from both progenitors, we targeted decrease in DNA methylation 1 (*DDM1*) because loss of *DDM1* activity causes an easily assayed decrease in centromere methylation (Vongs *et al.*, 1993). We show that expression in *A. suecica* of double-stranded (ds) RNA corresponding to a portion of *A. thaliana* *DDM1* induces de-methylation of both *A. thaliana*-like and *A. arenosa*-like centromere repeats and elimination of both *A. thaliana*- and *A. arenosa*-derived *DDM1* mRNAs. These data demonstrate that RNAi is an effective strategy for circumventing problems of gene redundancy, facilitating the generation of dominant loss-of-function mutations in a polyploid.

Results

Vernalization is needed for vigorous bolting in A. suecica

Arabidopsis suecica is increasingly studied within the research community, but its growth conditions are not well established. We therefore needed to identify conditions conducive to bolting and flowering so that we could attempt the floral dip method for genetic transformation. By systematically testing a variety of conditions, including day length, light intensity, and temperature regimes, we found that vernalization of 1–4-month-old *A. suecica* plants for 2 weeks at 4°C (under 24 h day⁻¹ fluorescent light) induces vigorous bolting. Following cold treatment, plants returned to the greenhouse or growth chamber bolt approximately 4 weeks later, regardless of the chronologic age of the plants. If vernalization is omitted, plants will eventually flower, usually after 4–6 months of growth, but the plants will produce small bolts with few flowers and produce few seeds (see Figure 1).

(a) Effect of vernalization



Untreated Cold-treated

(b) Closeups of non-vernalized plants

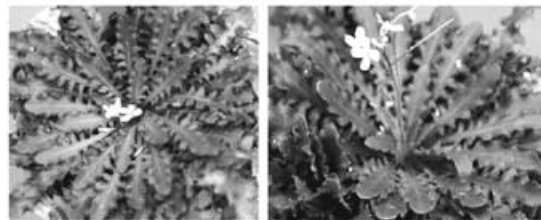


Figure 1. Effect of vernalization on bolting and flowering in *A. suecica*. Seeds for the four plants shown were sown on the same day and were then germinated and grown under short-day conditions in a growth chamber. After 15 weeks, the two plants on the left were moved to the greenhouse and the two plants on the right were moved to a 4°C-cold room under continuous fluorescent white light. After 2 weeks of cold treatment, the two plants on the right were moved to the greenhouse to join the other two plants. The photo was taken 10 weeks later, when all four plants were 27 weeks old. The seed-to-seed time-span can be decreased by 11 weeks by cold-treating plants when they are 4 weeks of age (not shown).

Transformation of A. suecica using the floral dip method

Plants destined for transformation were sown in soil and grown for approximately 4 weeks in a growth chamber, cold-treated for 2 weeks at 4°C, and then returned to the growth chamber. Approximately 1 week after the plants began to bolt (week 11 after planting); when secondary bolts were emerging from the rosette, the plants were dipped (Clough and Bent, 1998) into a log-phase culture of *A. tumefaciens* strain GV3101, harboring a binary plasmid vector for T-DNA transfer. Within the T-DNA, a hygromycin-resistance gene served as the selectable marker (see Figure 2a). The RNAi transgene within the T-DNA was a 492-bp portion of *DDM1* (nucleotide positions 1801–2293 relative to the adenosine in the initiation codon), cloned as an inverted repeat downstream of the cauliflower mosaic virus 35S promoter. Expression of the transgene should result in an RNA that folds back on itself to form a ds hairpin. Such dsRNAs are triggers for the RNAi machinery,

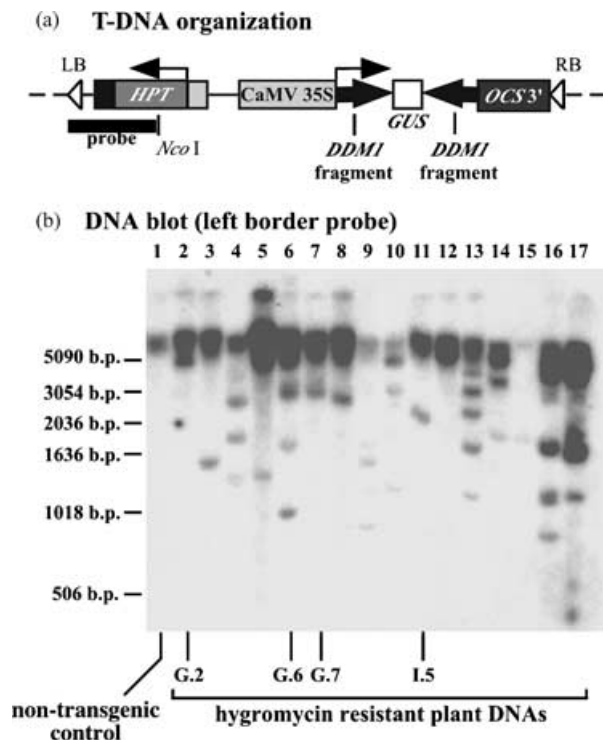


Figure 2. Transformation of *A. suecica* with a *DDM1*-RNAi construct. (a) Schematic diagram of the *DDM1*-RNAi binary vector. The directions of transcription for the *HPT* gene, conferring hygromycin resistance, and for the *DDM1* inverted repeat gene, are indicated by thin arrows. The cauliflower mosaic virus 35S promoter drives expression of the 492-bp *DDM1* inverted repeats (indicated by thick arrows), which are separated by a spacer derived from the *GUS* gene. The 3' end of the octopine synthase gene provides polyadenylation and termination signals for the *DDM1* inverted repeat gene. LB and RB refer to the left and right borders of the T-DNA, respectively. The *NcoI* site near the LB, cleaved in subsequent DNA blot analyses, is indicated. The black bar indicates the portion of the T-DNA that is identical to the DNA blot hybridization probe used in Figure 3(b). (b) DNA blot hybridization using DNA samples from control (lane 1) and transgenic (lanes 2–17) *A. suecica* *DDM1*-RNAi T_1 plants. All genomic DNAs were digested with *NcoI* and probed with a fragment corresponding to the LB region of the T-DNA. The four plants (G.2, G.6, G.7, and I.5), whose centromere methylation patterns are shown in Figure 3, are indicated. Note that there is cross-reaction between the probe and genomic DNA of non-transformed *A. suecica* (see lane 1), which can account for the uppermost band in all lanes.

which subsequently targets for degradation any mRNA with high similarity to the trigger RNA (Hannon, 2002; Vaucheret *et al.*, 2001). After dipping, the plants were returned to the growth chamber and allowed to set seed.

Five *A. suecica* plants were dipped into *A. tumefaciens* harboring the *DDM1*-RNAi vector. Mature seeds were harvested from the plants, sterilized, and sown on a medium containing hygromycin to select transformants. Nineteen hygromycin-resistant T_1 plants were selected and were obtained from the five parent (T_0) plants at a frequency of 1/1068 (0.1%), 4/1083 (0.4%), 0/147 (0%), 7/838 (0.8%), and 7/831 (0.8%), respectively. The average of 0.4% was similar to the frequency of *A. thaliana* transformation obtained using the floral dip method (Clough and Bent, 1998).

Verification of genetic transformation

DNA blot hybridization was used to verify that hygromycin-selected plants were genetically transformed and to estimate the number of T-DNAs integrated within their genomes (Figure 2b). Genomic DNA was digested with *NcoI*, which cuts once within the T-DNA near the left border, and blots were hybridized with a probe corresponding to the left-border region. Resulting bands on DNA blots should be unique in size because of the variable distance from the *NcoI* site within the T-DNA to the nearest *NcoI* site in the flanking genomic DNA. The appearance of multiple bands indicates that multiple T-DNAs are present. As shown in Figure 2(b), most lines contain multiple T-DNAs (as is also the case when *A. thaliana* is transformed using *A. tumefaciens* strain GV3101; data not shown). Upon testing the T_2 progeny of line I.5, which shows only one T-DNA restriction fragment (Figure 2b, lane 11), 44 T_2 plants were found to be resistant to hygromycin and 14 were hygromycin-sensitive. This diploid-like 3 : 1 segregation of the dominant marker gene is consistent with the hypothesis that in *A. suecica*, the *A. thaliana*-like chromosomes pair with their *A. thaliana*-like sister chromosomes and the *A. arenosa*-like chromosomes do likewise, such that any given gene displays diploid-like meiotic behavior.

Loss of *DDM1* function in transgenic *A. suecica*

DDM1 is required to maintain high levels of cytosine methylation at repetitive elements, including the 180-bp repeats that surround the centromeres of *A. thaliana* (Vongs *et al.*, 1993). Decreases in cytosine methylation at the centromere repeats is easily assayed by examining their sensitivity to digestion by *HpaII* and *MspI*. Both restriction endonucleases recognize the same sequence, CCGG, but differ in their sensitivity to methylation of the inner cytosine. *HpaII* will not cut if the inner C is methylated, whereas *MspI* will cut regardless of the methylation status of the inner cytosine (McClelland *et al.*, 1994).

Radioactive probes were made from the 180-bp *A. thaliana* centromere repeats or the *A. arenosa* 172-bp centromere repeats, and hybridized to DNA blots. At high stringency, the *A. thaliana* probe hybridizes to uncut genomic DNA of *A. thaliana* (Figure 3a, lane 1) and *A. suecica* (lane 2), but not to *A. arenosa* genomic DNA (lane 3; the faint signal is because of spill-over from an adjacent lane). Likewise, the *A. arenosa* centromere probe is also specific, hybridizing to genomic DNA of *A. suecica* and *A. arenosa* (Figure 3b, lanes 2 and 3), but not to *A. thaliana* DNA (Figure 3b, lane 1). In non-transformed siblings of transgenic *A. suecica* allotetraploids, both the *A. thaliana*-like and *A. arenosa*-like centromere repeats are heavily methylated such that they are resistant to digestion with *HpaII* (Figure 3a,b, lanes 4 and 6), but can be cleaved by *MspI*

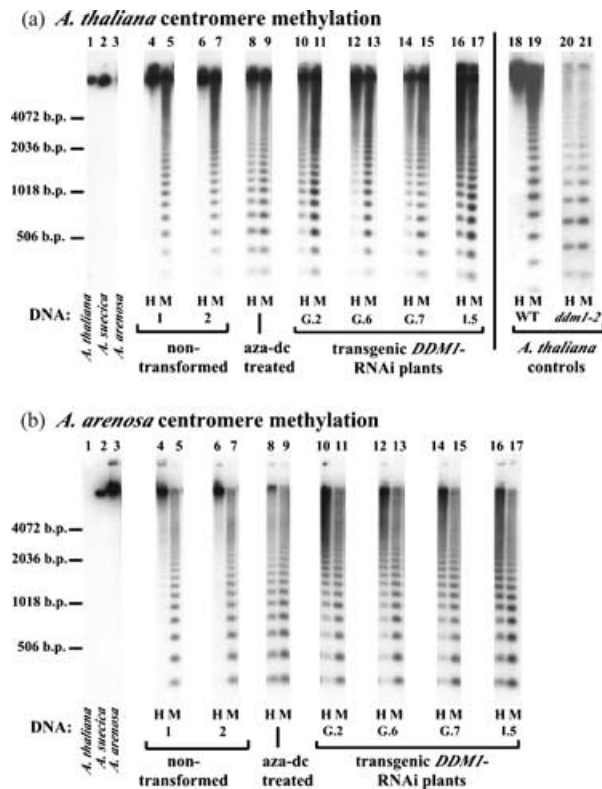


Figure 3. Centromere de-methylation in *A. suecica* *DDM1*-RNAi lines. (a) DNA samples from transgenic *A. suecica* *DDM1*-RNAi lines digested with *Hpa*II (H) or *Msp*I (M) and hybridized with an *A. thaliana*-specific centromere probe. Lanes 1–3 are *A. thaliana*-, *A. suecica*-, and *A. arenosa*-undigested genomic DNA controls that demonstrate the specificity of the *A. thaliana* centromere probe. Lanes 4–7 are DNA samples derived from non-transformed *A. suecica* plants digested with *Hpa*II and *Msp*I. Lanes 8–9 are DNA samples derived from 5'-aza-2'-deoxycytidine (aza-dC)-treated *A. suecica* digested with *Hpa*II and *Msp*I. Lanes 10–17 are DNA samples derived from transgenic *A. suecica* *DDM1*-RNAi lines digested with *Hpa*II and *Msp*I. For comparison, *Hpa*II- and *Msp*I-digested DNAs from *A. thaliana* wild-type and *ddm1-2* mutant plants are shown in lanes 18–21. (b) DNA blot of the same DNA samples indicated in panel 'a' (with the exception of *A. thaliana* controls) following hybridization to an *A. arenosa*-specific centromere probe.

(Figure 3a,b, lanes 5 and 7). In 2-week-old plants grown on sterile medium containing the cytosine methylation inhibitor 5'-aza-2'-deoxycytosine, methylation is lost from the centromere repeats such that they are readily cut by both *Hpa*II and *Msp*I (compare lanes 8 and 9 in Figure 3a,b). Centromere methylation is also decreased in *A. suecica* plants transgenic for the *DDM1*-RNAi construct (Figure 3a,b, lanes 10–17), as indicated by the ability of *Hpa*II to cut the centromere repeats inherited from both progenitor species, yielding a digestion pattern similar to that of a homozygous *ddm1-2* mutant in *A. thaliana* (lanes 20 and 21). We conclude that expression of an RNAi construct made using the *A. thaliana* *DDM1* gene is sufficient to knock down *DDM1* activity in allotetraploid cells.

The simplest interpretation for the results of Figure 3 is that loss of *DDM1* activity as a result of RNAi is because of

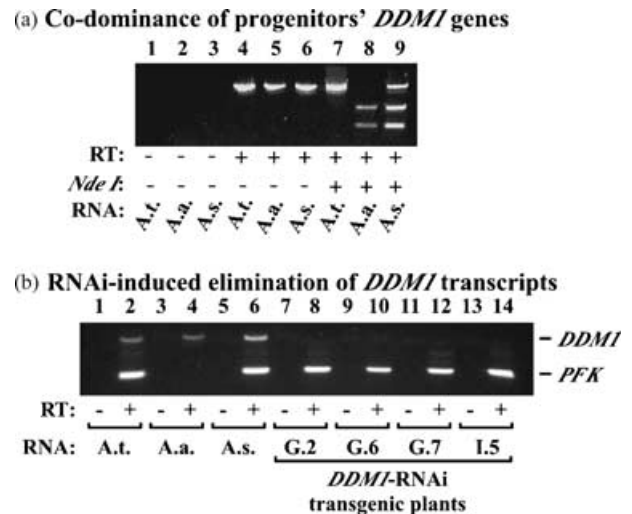


Figure 4. *DDM1* mRNA is undetectable in *DDM1*-RNAi transgenic plants. (a) RT-PCR detection of *A. thaliana* and *A. arenosa* *DDM1* transcripts in *A. suecica* reveals co-dominance of both progenitors' *DDM1* genes. Total RNA isolated from *A. thaliana* (A.t.), *A. arenosa* (A.a.), or *A. suecica* (A.s.) was reverse transcribed (RT; lanes 4–9), and the resulting DNA was subjected to PCR using a single primer pair (see Figure 5 for primer locations). RT-PCR products were then subjected to agarose gel electrophoresis either with (lanes 7–9) or without (lanes 4–6) prior digestion by the restriction endonuclease *Nde*I, which cuts the *A. arenosa* but not the *A. thaliana* *DDM1* gene (see Figure 5 for the polymorphism). Note that both *A. thaliana* and *A. arenosa* transcripts are detected in *A. suecica* (lane 9). In lanes 1–3, reverse transcriptase was omitted prior to PCR; these controls show that RNA samples were not contaminated with genomic DNA. (b) RT-PCR analysis of *DDM1* transcript levels in control and *DDM1*-RNAi transgenic plants. Even-numbered lanes show PCR products following reverse transcription and amplification of cDNAs using primers specific for *DDM1* and the PFK β -subunit (lower band). Odd-numbered lanes are equivalent reactions from which reverse transcriptase was omitted to show that genomic DNA does not contaminate the RNA samples. Lanes 2, 4, and 6 show RT-PCR products using RNA isolated from *A. thaliana*, *A. arenosa*, or non-transgenic *A. suecica*, respectively. Note that the PFK primers, designed using the *A. thaliana* PFK gene sequence, do not amplify *A. arenosa* PFK mRNA(s). *DDM1* transcripts are undetectable in *A. suecica* *DDM1*-RNAi transgenic plants G.2, G.6, G.7, and I.5 (lanes 8, 10, 12, and 14, respectively).

depletion of transcripts from both progenitors' *DDM1* genes. However, because there is evidence that a small percentage of orthologous genes in *A. suecica* are expressed uniparentally (Comai *et al.*, 2000; Lee and Chen, 2001), an alternative possibility was that the *A. arenosa* *DDM1* gene was already silenced because of polyploidization, leaving only the *A. thaliana* *DDM1* gene to be targeted by RNAi. So, to determine if both progenitors' *DDM1* genes are expressed in *A. suecica*, we devised an assay using reverse transcription followed by PCR (RT-PCR) and digestion with a restriction endonuclease that could distinguish the transcripts (Figure 4a). Using a single pair of primers, a *DDM1* RT-PCR product of identical size was generated with RNA isolated from *A. thaliana*, *A. arenosa*, or *A. suecica* (Figure 4a, lanes 4–6). However, an *Nde*I site present in the *A. arenosa* *DDM1* gene, but missing in the *A. thaliana*

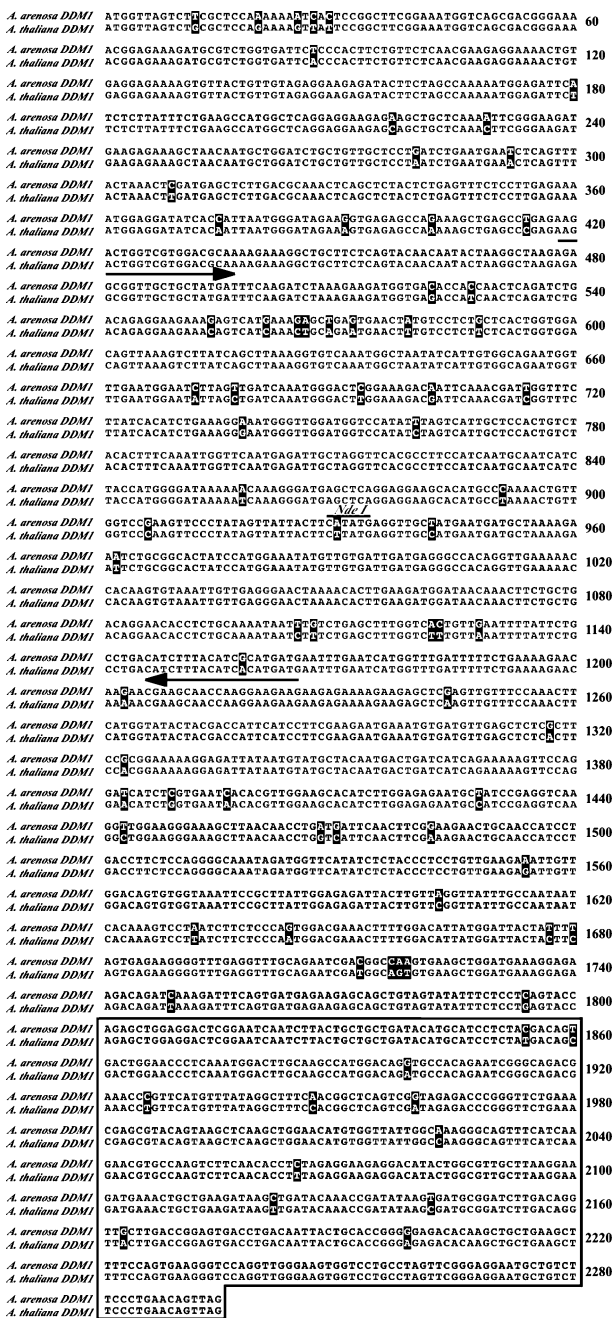


Figure 5. Comparison of *A. thaliana* (ecotype Columbia) and *A. arenosa* *DDM1* ORFs highlighting sequences relevant to this study. The sequences are aligned beginning with the initiating methionine and ending with the TAG stop codon. Nucleotide differences are shaded. Arrows denote the locations of the PCR primers that bracket a polymorphic *Nde*I site (position approximately 930) used to discriminate *A. thaliana* and *A. arenosa* transcripts in Figure 4. The boxed region at the 3' end of the ORF corresponds to the sequences used in each of the inverted repeats of the RNAi-inducing transgene.

gene, allowed RT-PCR products of the orthologs to be discriminated (lanes 7 and 8). As can be seen in lane 9, both the *A. thaliana*- and *A. arenosa*-derived *DDM1* genes are expressed in *A. suecica*.

Having established the co-identity of *A. thaliana*- and *A. arenosa*-derived *DDM1* genes in *A. suecica*, we examined the effectiveness of RNAi in reducing *DDM1* mRNA levels (Figure 4b). Using the same primer pair used in Figure 4(a), *DDM1* RT-PCR products were readily detected in *A. thaliana*, *A. arenosa*, and non-transgenic *A. suecica* (lanes 2, 4, and 6, respectively). The RT-PCR product for the beta subunit of the *A. thaliana* phosphofructokinase (PFK) gene served as an internal control in these reactions (note that *A. arenosa* PFK is not amplified by these primers in lane 4). However, in the *A. suecica* plants bearing the *DDM1*-RNAi transgene, no *DDM1* RT-PCR products could be detected (lanes 8, 10, 12, and 14). Taken together with the results of Figure 4(a), these data show that both the *A. thaliana*- and *A. arenosa*-derived *DDM1* mRNAs were efficiently knocked down by RNAi.

Our transgenic plant production and *DDM1* expression studies were conducted without knowing the degree of sequence identity shared by the *A. thaliana* and *A. arenosa* *DDM1* genes. For the sake of completeness, we sequenced the *A. arenosa* *DDM1* gene and aligned its deduced open reading frame (ORF) with that of its ortholog from *A. thaliana* ecotype Columbia (Figure 5). Overall, the coding sequences are 96% identical. Within the 3'-region corresponding to the inverted repeats of the RNAi-inducing transgene (boxed region in Figure 5), intervals of 100% identity range from 5 to 95 nucleotides and average 37 nucleotides in length. Trigger dsRNAs as short as 23–26 bp can induce degradation of target mRNAs (Parrish *et al.*, 2000; Thomas *et al.*, 2001). Thus, the *A. thaliana* and *A. arenosa* *DDM1* genes clearly possess sufficient regions of identity for a single trigger dsRNA to target its ortholog(s) in *A. suecica*.

Discussion

Arabidopsis suecica is an emerging model system for investigating the genetic, epigenetic, and phenotypic consequences of polyploidization (Chen *et al.*, 1998; Comai *et al.*, 2000; Lee and Chen, 2001; Madlung *et al.*, 2002) and is a promising system for studying vernalization physiology. An ability to genetically transform *A. suecica* is highly desirable, as this capability opens up new avenues of inquiry concerning gene function. As we demonstrate in this report, *A. suecica* can be genetically transformed using the floral dip method that is commonly used to transform *A. thaliana*. The efficiency of transforming *A. suecica* using this simple method is similar to the efficiency of transforming *A. thaliana*, and averaged 0.4% in our study.

A useful observation in our study is that the dominant hygromycin-resistance trait encoded by a single-copy transgene in a T₁ (first transformed generation) plant is transmitted to the T₂ progeny in a 3 : 1 resistant:sensitive ratio. This is the expected phenotypic ratio for a dominant

gene segregating in a diploid. Such genetic behavior is common among natural allopolyploids as a result of pairing of homologous chromosomes and suppression of pairing among homeologous chromosomes. Cytogenetic evidence generally supports the transgene segregation ratio we observed. For instance, using species-specific centromere probes, one does not observe *A. thaliana*- and *A. arenosa*-derived chromosomes paired non-homologously at metaphase in *A. suecica* mitotic cells (Pontes *et al.*, data not shown). However, in meiotic cells, occasional multivalents and lagging chromosomes have been observed, suggesting that homolog-only pairing may be the rule but is not absolute in *A. suecica* (Comai *et al.*, 2000).

By introducing a transgene expressing a ds RNA corresponding to the 3' end of the *A. thaliana* *DDM1* mRNA, we demonstrate the loss of centromere methylation in *A. suecica*. DNA blot analysis showed that the T-DNAs bearing the *DDM1* RNAi transgenes were integrated into the *A. suecica* genome in one to several copies. As shown in Figures 3 and 4, the degree to which centromere repeats were de-methylated and *DDM1* mRNAs were knocked down, respectively, was similar in all the transgenic plants, revealing that multiple transgene copies conferred no advantage over single-copy transgenes in terms of triggering RNAi. Given that fact, single-copy transgenes may be advantageous because of the propensity of multi-copy transgenes to become transcriptionally silenced (Muskens *et al.*, 2000; Stam *et al.*, 1998), in which case production of the dsRNAs to trigger target gene degradation would cease.

Our data indicate that RNAi is a viable method for causing dominant loss-of-function mutations in a polyploid species. In addition to successfully knocking down *DDM1*, we have also used RNAi vectors targeting chromatin-modifying genes to eliminate target mRNAs and yield mutant phenotypes in *A. suecica* (Lawrence and Pikaard, data not shown). Furthermore, in cotton, which is an allotetraploid, there is a brief report that expression of inverted-repeat transgenes targeting seed-specific fatty acid desaturases caused the expected changes in fatty-acid profiles (Liu *et al.*, 2000). Unfortunately, no molecular data were provided in the cotton seed study to show that both progenitors' orthologs were retained in the allotetraploid, were co-expressed, or were equally targeted by RNAi. A final consideration is that a recent microarray study of RNA profiles in cultured human cells revealed that genes containing as few as 11 contiguous nucleotides identical to a trigger RNA can be silenced by RNAi (Jackson *et al.*, 2003). Given the likelihood that orthologous genes will retain such minimal regions of identity, RNAi should be an effective strategy for generating loss-of-function phenotypes in numerous polyploids for which sequence information is available for only one ortholog.

Experimental procedures

A. suecica growth and transformation

Arabidopsis suecica plants (lab strain LC1) were grown for 4 weeks under long-day conditions (16-h light/8-h dark, 21°C, 70% humidity) and then transferred to a 4°C-cold room for 2 weeks. During cold treatment, plants were continuously illuminated (24 h) with two 40-W cool-white fluorescent tubes suspended approximately 50 cm above the plants. Vernalized plants were returned to the growth chamber and typically bolted in approximately 4 weeks. One week after the bolts were initiated, the plants were transformed using published methods (Clough and Bent, 1998) and *A. tumefaciens* strain, GV3101, harboring the *DDM1*-RNAi vector pFGC1632 (supplied by Dr Carolyn Napoli, University of Arizona). Briefly, a 600-ml culture of *A. tumefaciens* was grown overnight in Luria-Bertani medium, pelleted, and re-suspended in 600 ml of 5% sucrose, 0.02% Silwet L-77. *A. suecica* plants were immersed in this solution for 5 min, and then were placed horizontally and incubated overnight in a tray covered with a transparent plastic lid before being returned to the growth chamber. Seeds harvested from mature plants were sterilized and sown on MS agar plates containing 15 µg ml⁻¹ hygromycin. Putative transgenic plants were selected after 2 weeks of growth on hygromycin plates, and they differed from non-transformed plants by the ability of their roots to elongate and true leaves to develop. Putative transformants were transplanted onto soil and grown under long-day conditions in a growth chamber.

The *A. suecica* seedlings, treated with 5'-aza-2'-deoxycytidine, were sterilized and sown on MS agar plates supplemented with 10 µg ml⁻¹ 5'-aza-2'-deoxycytidine. The plates were cold-treated at 4°C for 48 h and then transferred to a growth chamber where they were grown under continuous light for 14 days.

DNA blot analysis

The transgenic nature of hygromycin-resistant plants was confirmed by DNA blot analysis. Three micrograms of genomic DNA was digested with *Nco*I and subjected to electrophoresis on a 1% TBE-agarose gel. The DNA blot was then hybridized with a radioactively labeled probe corresponding to the T-DNA left border, which was the DNA fragment isolated from plasmid vector pFGC5764 (supplied by Dr Carolyn Napoli, University of Arizona), following digestion with *Pst*I and *Nco*I. For centromere methylation analysis, 1 µg of DNA was digested with *Hpa*II or *Msp*I, subjected to electrophoresis, blotted, and hybridized with radioactively labeled pARR20-1 (Feinberg and Vogelstein, 1983), which contains an *A. thaliana* centromere repeat (provided by Dr Eric Richards). *A. arenosa* centromeres were detected with a probe generated using PCR with the following primers: 5'-AGCTTCTTATTGCTCTCAACGG-3' and 5'-TTAGAAGCTCCAAAACCGAAAA-3'. Cycling conditions were: 94°C for 2 min followed by 40 cycles of 94°C for 25 sec, 57°C for 30 sec, and 72°C for 45 sec. The 236-bp PCR product was then radioactively labeled using the random priming technique (Feinberg and Vogelstein, 1983).

RT-PCR analysis

RT-PCR was carried out as previously described by Lewis and Pikaard (2001). Briefly, 250 ng of LiCl-precipitated RNA was treated with 2 units of RQ1 DNase (Promega Corporation, Catalog No. M6101) in a 10-µl reaction for 30 min at 37°C to remove any residual DNA. The DNase reaction was stopped by addition of

1 µl of RQ1 'stop' buffer (Promega) and by heat inactivation at 65°C for 10 min. Five microliter of DNA-free RNA (approximately 110 ng) was reverse-transcribed using 200 units of SuperScript II RNA-dependent DNA polymerase (Invitrogen Life Technologies, Catalog No. 18064) in a 20-µl reaction incubated at 42°C for 50 min followed by 85°C for 5 min. Mock reactions lacked Superscript II. One microliter of resulting cDNA was used in a PCR reaction designed to detect a 748-bp fragment of *DDM1* (forward primer: 5'-AGAGCGGTTGCTGCTATGAT-3'; reverse primer: 5'-CTTCTTC-CTTGGTTGCTCG-3') and an approximately 400-bp fragment of the PFK β-subunit (forward primer: 5'-GCCACGAAAACCAACAG-AC-3'; reverse primer: 5'-CCGGAATTCGATCAATCCT-3') control. PCR reactions were incubated at 95°C for 2 min and were then subjected to 40 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 90 sec. A final incubation at 72°C was for 10 min.

DDM1 sequence analysis

Overlapping segments of the *A. arenosa* *DDM1* gene, each approximately of 500–700 bp, were amplified by PCR using purified genomic template DNA and eight primer pairs (primer sequences available upon request). PCR products were cloned into a Topo-TA vector (Invitrogen), and several clones of each *DDM1* segment were sequenced on both strands using Big Dye Terminator reagents (Applied Biosystems, Catalog No. 4337455) and an ABI3700 sequencer. Sequences were assembled into a single contiguous sequence using SeqMan (DNASTAR Inc.). Exons were identified by comparison to the *DDM1* sequence of *A. thaliana* ecotype Columbia (GenBank accession number AF143940). ORFs were aligned and annotated using BIOLOGY WORKBENCH (version 3.2) software tools available via the internet (<http://workbench.sdsc.edu/>). The *A. arenosa* *DDM1* gene sequence has been deposited at GenBank (Accession number AY333120).

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