Unusual Case of Apparent Hypermutation in Arabidopsis thaliana

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ABSTRACT The dms4 (defective in meristem silencing 4) mutant of Arabidopsis thaliana is unique in having defects in both RNA-directed DNA methylation (RdDM) and plant development. DMS4 is an evolutionarily conserved, putative transcription factor of the Iwr1 (interacts with RNA polymerase II) type. DMS4 interacts with Pol II and also with RNA polymerases IV and V, which function in RdDM. Interactions with multiple polymerases may account for the diverse phenotypic effects of dms4 mutations. To dissect further the roles of DMS4 in RdDM and development, we performed a genetic suppressor screen using the dms4-1 allele, which contains in the sixth intron a splice site acceptor mutation that alters splicing and destroys the open reading frame. Following mutagenesis of dms4-1 seeds using ethyl methanesulfonate (EMS), we retrieved four dominant intragenic suppressor mutations that restored DMS4 function and wild-type phenotypes. Three of the four intragenic suppressor mutations created new splice site acceptors, which resulted in reestablishment of the wild-type open reading frame. Remarkably, the intragenic suppressor mutations were recovered at frequencies ranging from 35 to 150 times higher than expected for standard EMS mutagenesis in Arabidopsis. Whole-genome sequencing did not reveal an elevated mutation frequency genome-wide, indicating that the apparent hypermutation was confined to four specific sites in the dms4 gene. The localized high mutation frequency correlated with restoration of DMS4 function implies an efficient mechanism for targeted mutagenesis or selection of more fit revertant cells in the shoot apical meristem, thereby rapidly restoring a wild-type phenotype that is transmitted to future generations.

RNA-directed DNA methylation (RdDM) is a small RNA-mediated epigenetic modification that contributes to transcriptional silencing of transposons and repetitive sequences in plants. Forward genetic screens have retrieved a number of specialized factors required for RdDM, including subunits of atypical RNA polymerases, called Pol IV and Pol V, as well as chromatin remodelers, transcription factors, RNA binding proteins, and other novel factors whose precise functions in the RdDM pathway are unclear (Matzke et al. 2009; Haag and Pikaard 2011). Despite the presumed role of RdDM in transposon silencing, most mutants defective in this process do not mobilize transposons nor do they display overt developmental phenotypes. An exception is dms4 (defective in meristem silencing 4), which is the only mutant identified so far that is impaired in both RdDM and plant development (He et al. 2009; Kanno et al. 2010).

We identified dms4 mutations in a forward genetic screen for mutants deficient in RdDM and transcriptional gene silencing of a GFP reporter gene in shoot and root meristem regions in Arabidopsis thaliana (Arabidopsis) (Kanno et al. 2008, 2010). In addition to reducing RdDM and releasing GFP silencing, mutations in DMS4 condition a pleiotropic developmental phenotype characterized by late seed germination, dwarf stature, pale and serrated leaves, late flowering, small flowers, and abnormal phyllotaxy (Kanno et al. 2010).

DMS4, identified as RDM4 in a separate genetic screen (He et al. 2009), is an evolutionarily conserved, putative transcription factor of the Iwr1 type (interacts with RNA polymerase II). Iwr1 was first identified in a global proteomics analysis in budding yeast, where it was shown to interact with
many subunits of RNA polymerase II (Pol II) (Collins et al. 2007). Recent work in budding yeast has demonstrated that Iwr1 is required for nuclear import of Pol II (Czeko et al. 2011) and for transcriptional initiation by Pols I, II, and III (Esberg et al. 2011). In Arabidopsis, DMS4 has been shown to interact with Pol II (He et al. 2009) and also with Pol IV (Law et al. 2011) and Pol V (He et al. 2009). The diverse phenotypic effects of dms4 mutations may thus reflect interactions with multiple RNA polymerases (He et al. 2009; Kanno et al. 2010). However, the precise function of DMS4 in various Pol II-, Pol IV-, and Pol V-dependent processes remains unknown.

To dissect the roles of DMS4 in RdDM and development, we have carried out a genetic suppressor screen in Arabidopsis to identify second site mutations that either restore RdDM/GFP silencing, normal development, or both. All three categories of mutant have been retrieved in this screen, demonstrating that it is possible to genetically separate the effects of a dms4 mutation on RdDM and development. Here we report on four dominant, intragenic suppressor mutations that reestablish both RdDM and normal development. Remarkably, these intragenic suppressor mutations were observed at frequencies that range from ~35 to 150 times higher than normally observed with standard EMS mutagenesis in Arabidopsis. We discuss this unexpectedly high mutation frequency in the context of targeted mutagenesis of the dms4 gene or selection of more fit revertant cells in the shoot apical meristem.

Materials and Methods

Plant materials and EMS mutagenesis

The two-component transgene silencing system (T+S) used to identify the dms4-1 mutant is described in previous publications (Kanno et al. 2008, 2010) and illustrated in Supporting Information, Figure S1. The Arabidopsis gene identifier number of DMS4 is AT2g30280. Approximately 14,400 BC1F3 seeds of dms4-1 (Kanno et al. 2010) were mutagenized by treatment with 1% EMS (Sigma-Aldrich) for 3 hr. Mutagenized seeds (M1 generation) were sown on soil and grown in a growth chamber at 21°C under a 16-h light/8-h dark regime. Approximately 10,600 M1 plants grew from the mutagenized seed. M1 plants with a wild-type–like phenotype were identified, the dms4 gene was sequenced, and self-pollinated seeds (M2 generation) were harvested from each individual M1 plant showing a wild-type phenotype. All other M1 plants were pooled into 144 batches (containing ~74 M1 plants/batch), and the M2 seeds were harvested in bulk from each batch. Approximately 400–500 M2 seedlings per batch (~5–7 M2 seedlings from each M1 plant) were sown on solid Murashige and Skoog (MS) medium and GFP–plants were screened using a Leica fluorescence stereomicroscope. For the second EMS mutagenesis treatment, ~5500 BC2F4 dms4-1 seeds were treated as described above, and M2 seeds from ~3575 M1 plants were harvested and screened for GFP. “BC” refers to a backcross of the dms4 mutant to the wild type T+S line, with the subscript number indicating the number of times the backcross has been carried out. “F” refers to generations of self-fertilization following a backcross.

DNA methylation analyses

Genomic DNAs were extracted from mature rosette leaves using DNeasy Plant Mini kit (Qiagen). We analyzed methylation of endogenous sequences by Chop–PCR, an assay in which genomic DNA is digested (“chopped”) with a methylation-sensitive restriction enzyme, and then used as a template for PCR amplification using primers flanking the restriction enzyme site (Earley et al. 2010). For this, 50 ng of genomic DNA was digested with HaeIII, which reports on CHH methylation (H is A, T, or C), in 20 μl of reaction mix at 37°C overnight. After restriction digestion, 1 μl of digested DNA was used as template for PCR in the 10-μl reaction mix. Primers used for Chop–PCR are listed in Table S1.

For the bisulfite sequencing analysis of the target enhancer region, 1 μg of genomic DNA, isolated from rosette leaves as described above, was digested with HaeIII, and then 500 ng was used for bisulfite conversion of unmethylated cytosines to uracil using the EpiTect Bisulfite kit (Qiagen). Amplified fragments were cloned using pGEM-T Easy Vector system (Promega) and 10–20 clones were sequenced. Complete conversion was confirmed by analyzing the methylation-free PHAVOLUTA (PHV) locus (Reinders et al. 2008). The primers used are shown in Table S1.

Analysis of dms4 transcription

Total RNA was extracted from rosette leaves using the RNeasy Plant Mini kit (Qiagen) and treated with TURBO DNA-free DNase (Ambion). Approximately 2.5–3.0 μg of total RNA was reverse transcribed using the ReverseAid H Minus First Strand cDNA Synthesis kit (Fermentas). RT–PCR products using primer pair 3028-3 and DMS4sqR2 (Table S1) were cloned using the pGEM-T Easy Vector system and sequenced.

Western blot analysis

Nuclei were extracted from the following lines: T+S, dms4-1, dms4-1s1, dms4-1s2, dms4-1s3, and dms4-1s4. Around 1.5 g of frozen rosette leaves were ground in liquid nitrogen and resuspended in 30 ml extraction buffer [0.4 M sucrose, 10 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 5 mM β-mercaptoethanol] supplemented with EDTA-free Proteinase Inhibitor cocktail (Roche). The suspension was centrifuged at 2,000 × g for 15 min at 4°C. The supernatant was discarded and the pellet was resuspended in 1 ml of extraction buffer 2 [0.25 M sucrose, 10 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 1% Triton-100, 5 mM β-mercaptoethanol] supplemented with EDTA-free proteinase inhibitor, and centrifuged at 2,000 × g for 10 min at 4°C. This step was repeated. The final pellet was resuspended in nuclei lysis buffer [50 mM Tris-HCl (pH 8.0),
Apparent Hypermutation in dms4 Mutant

10 mM EDTA, 1% SDS] supplemented with EDTA-free Proteinase Inhibitor, and sonicated 3 × 10 sec, 40% duty cycle, and 20% power. Proteins were separated by SDS–PAGE (10% gel), transferred to a PVDF membrane (Millipore), followed by Western blotting according to standard procedures. Rabbit anti-DMS4 polyclonal antibodies were generated by Eurogentec (Belgium) using overexpressed DMS4 protein in bacteria and used at 1:10,000 dilutions. Secondary antibody [goat antirabbit IgG-conjugated with horseradish peroxidase (Biorad)], was used at 1:10,000 dilution. The blots were developed using an enhanced chemiluminescence kit (AmershamPharmacia Biotech).

Whole-genome sequencing

Total DNA was extracted from 1.5 g of root cultures grown in liquid MS medium with shaking using the CTAB method (Eun et al. 2011). For each suppressor mutant, we isolated DNA from root cultures of approximately five pooled M_3 plants. Around 400 ng of DNA was sonicated using a Covaris S2 (Covaris, Woburn, MA) to produce fragments ~300–800 bp in length for making sequence libraries for paired-end reads.

Data analysis

Reads of whole-genome sequence were assembled to the Arabidopsis genomic sequence of TAIR9 (http://www.arabidopsis.org/) using CLC Genomics Workbench (CLC bio). To reduce the effect of sequencing errors, we discarded 5’- and 3’-end nucleotides of each read to remove low-quality bases. To reduce errors caused by presequencing amplification, we removed reads that had identical locations of starts and ends. Because each of the five plants used for DNA isolation was still segregating mutations induced by EMS during the suppressor screen, it was conceivable that at a minimum, only one of these plants still contained an induced mutation in the heterozygous state (i.e., in 10% of the pooled DNA isolated from five diploid plants). Therefore, mismatched nucleotides with more than five reads and >10% coverage were counted as single nucleotide polymorphisms (SNPs) induced by EMS mutagenesis. We focused on G/C to A/T changes, which typically result from EMS mutagenesis (Greene et al. 2003), and SNPs in suppressor mutants were compared with those in the T+S and dms4-1 lines. There were 319 common SNPs among the five suppressor mutants; however, 294 of them were already present in the original dms4-1 seed population and were therefore removed from the analysis. Of the remaining 25 SNPs, 15 were shown by sequencing to preexist in the mutagenized dms4-1 seed population and were therefore removed from the analysis. Of the final 10 common SNPs, 7 were within repetitive sequences, making it difficult to sequence a specific copy, and 3 were undetectable in the small population of dms4-1 seedlings tested. Nevertheless, the simplest explanation is that these 10 SNPs preexisted in the original dms4-1 seed population.

Results
dms4-1 suppressor screen: dominant intragenic suppressor mutations in M_1 plants

The scheme for the suppressor screen is illustrated in Figure 1A. Here we focus on mutations that restore both RdDM/
the dms4 gene in DNA isolated from leaves of 7 of these plants (the dms4 gene was sequenced in the remaining 12 in the M2 generation) (Table S2). All of the wild-type–appearing plants were homozygous for the original dms4-1 mutation and in addition, each was heterozygous for a second mutation within the dms4 gene. Four different G to A intragenic suppressor mutations, all within 200 bp of the original dms4-1 mutation, were identified (Figure 3A). Chimeric plants containing wild-type and dms4-like sectors were found to be genetic mosaics in which the heterozygous suppressor mutation was detected only in the wild-type leaves (Figure 2D).

Because EMS-induced mutations in the M1 generation are still heterozygous, all of the intragenic suppressor mutations that restore a wild-type phenotype act as dominant mutations. We named the suppressor mutations dms4-1s1, dms4-1s2, dms4-1s3, and dms4-1s4 (Figure 3A). These four intragenic suppressor mutations were recovered multiple times in the M1 population (Table 1). None of the suppressor mutations were observed following a mock treatment of dms4-1 seeds, confirming they did not preexist or occur spontaneously in the mutagenized seed population. Because each M1 plant was derived from an individually mutagenized seed, we could conclude that the suppressor mutations were induced independently multiple times during EMS mutagenesis.

All 19 of the wild-type–appearing M1 plants containing the dominant intragenic suppressor mutations spawned variable numbers of GFP−M2 progeny (Table S2), indicating that GFP silencing was restored in plants containing a suppressor mutation (Figure 4A). Consistent transmission to the M2 generation indicated that the dms4-1 suppressor mutations were present in germ cell progenitors in the L2 layer of the shoot apical meristem (SAM) of M1 plants.

M2 plants: inheritance of intragenic suppressor mutations and restoration of GFP silencing

Screening for GFP− seedlings in the M2 progeny of the remaining M1 plants (approximately five to seven M2 seedlings per M1 plant were sampled) identified additional cases of one of the four previously identified intragenic suppressor mutations (Table 1, Table S3). When transferred to soil and grown to maturity, all of the GFP−M2 plants lacked the developmental defects associated with the dms4-1 mutation. In addition to restoring normal development and GFP silencing, the dms4-1 suppressor mutations fully reestablished DNA methylation of the target enhancer driving GFP expression (Figure 4B) and several endogenous targets of RdDM (Figure 4C).

Because the intragenic suppressor mutations were identified in plants grown from separate batches of M2 seeds that did not contain seeds from the suppressor mutants identified in the M1 generation, they are believed to have arisen independently during EMS mutagenesis. In principle—given the dominant nature of the suppressor mutations—the parents of these M2 suppressor mutants could have been detected in the M1 generation but they were apparently

**Figure 2** Chimeric M1 suppressor plant. (A) Photographs of the wild-type (WT) T+S line, the dms4-1 mutant, and an M1 plant that is chimeric for a dominant intragenic suppressor mutation (dms4-1s1). This plant comprises a dms4-1–like sector (bottom red box) and a WT sector with normal flowers (top red box). Close ups of boxed regions are shown in B and C, respectively. (D) Sequencing chromatograms of the DMS4 gene in the WT sector (top) and dms4-1–like sector (bottom) of the chimeric M1 plant shown in A. Arrows indicate positions of the recessive dms4-1 mutation, which is homozygous in both sectors, and the dominant dms4-1s1 mutation, which is heterozygous in the WT sector.

GFP silencing and normal development (dominant sdm mutants, Figure 1A). For the screen, we used the dms4-1 allele, which has a mutation in the splice site acceptor of the sixth intron. This mutation disrupts the open reading frame, leading to an altered amino acid sequence in the final third of the DMS4 protein (Kanno et al. 2010). Approximately 14,400 dms4-1 seeds (BC1F2 generation) were treated with EMS according to standard protocols and sown on soil. As the M1 plants were bolting, flowering and setting seed, we noticed nineteen that had wild-type characteristics, being taller than dms4-1 mutants and displaying normal leaves, phyllotaxy, and flowers (Figure 1B). Several of these M1 plants appeared chimeric, containing both wild-type and dms4-1 sectors (Figure 2, A–C).

To rule out that these wild-type–appearing M1 plants were due to contamination by wild-type seeds, we sequenced
overlooked at that time, perhaps owing to the high density at which the M1 plants were grown.

**Nature of dms4-1 intragenic suppressor mutations**

Three of the suppressor mutations, dms4-1s1, dms4-1s2, and dms4-1s3, created new splice site acceptors that were predicted to reestablish the wild-type DMS4 open reading frame (Figure 3B). Sequencing of dms4 cDNAs synthesized from mRNA isolated from each suppressor mutant confirmed that the new splice site acceptors were used, although there was some sequence variability among the cDNA clones due to alternative splicing (Figure S2). Despite minor variations in the amino acid sequence arising from use of the new splice site acceptors (Figure 3C), the DMS4 proteins translated from the resulting cDNAs could be detected on Western blots using a DMS4 polyclonal antibody (Figure 3D) and were fully functional in RdDM/GFP silencing and development, as demonstrated by the phenotypic analyses described above. One suppressor mutation, dms4-1s4, did not create a new canonical splice site acceptor site (Figure 3A) although a rare cDNA containing a restored reading frame (Figure S2). Bottom shows the stained membrane as a loading control.

![Figure 3](Image)

**Table 1 Mutation frequencies of suppressors**

| Allele      | M1 generation | M2 generation | Total | Mutation frequency | Poisson |
|-------------|---------------|---------------|-------|--------------------|---------|
| First mutagenesis (10,599) | | | | | |
| dms4-1s1    | 10            | 17            | 27    | 2.5 × 10⁻³         | 3.1E-43 |
| dms4-1s2    | 4             | 6             | 10    | 9.4 × 10⁻⁴         | 1.0E-12 |
| dms4-1s3    | 4             | 4             | 8     | 7.5 × 10⁻⁴         | 1.0E-09 |
| dms4-1s4    | 1             | 5             | 6     | 5.7 × 10⁻⁴         | 6.7E-07 |
| Total       | 19            | 32            | 51    |                    |         |
| Second mutagenesis (3575) | | | | | |
| dms4-1s1    | 7             | 1             | 8     | 2.2 × 10⁻³         | 2.1E-13 |
| dms4-1s2    | 1             | 0             | 1     | 2.8 × 10⁻⁴         | 9.0E-02 |
| dms4-1s3    | 4             | 0             | 4     | 1.1 × 10⁻³         | 3.7E-06 |
| dms4-1s4    | 2             | 2             | 4     | 1.1 × 10⁻³         | 3.7E-06 |
| Total       | 14            | 3             | 17    |                    |         |

Two rounds of EMS mutagenesis were carried out on dms4-1 seeds of the BC1F3 and BC2F4 generations, respectively. The number of M1 plants obtained following each mutagenesis treatment is shown in parentheses. The number of times each suppressor mutation was identified in the M1 and M2 generations is indicated together with the mutation frequencies (calculated as the total number of plants having a mutation divided by the size of the M1 population). The average mutation frequencies in the first and second round are 1.2 × 10⁻³ and 1.17 × 10⁻³, respectively. Poisson probability indicates the probability of recurrent suppressor mutations. λ is calculated as (1189.8 (average number of induced mutations) × 10,599 or 3575 (mutagenized population)/42,859,753 (GCs in Arabidopsis genome).
High frequency of suppressor mutations

A total of 51 independent cases of the four dms4-1 suppressor mutations were recovered from an initial population of ~10,600 M1 plants (Table 1). The individual mutation frequencies (the number of times a mutation is observed divided by the size of the M1 population) (Jander et al. 2003) ranged from $2.5 \times 10^{-3}$ for dms4-1s1 to $5.7 \times 10^{-4}$ for dms4-1s4. The average mutation frequency was $1.2 \times 10^{-3}$ (Table 1). These frequencies can be compared to those found in a previous case of saturation EMS mutagenesis in Arabidopsis, where the average frequency of mutations in the CSR (CHLORSULFURON/IMIDAZOLINONE RESISTANT 1) gene leading to herbicide resistance was determined (Jander et al. 2003). The CSR gene, which is 2700 bp in length and free of introns, encodes the catalytic subunit of acetolactate synthase (ALS). Four amino acid substitutions resulting from distinct point mutations in the CSR gene prevent binding of various ALS-inhibiting herbicides and hence confer herbicide resistance. The average mutation frequency observed in that study was $1.6 \times 10^{-5}$ (Jander et al. 2003). The mutation frequencies we observed are thus ~35–150 times higher than reported previously for a case of standard EMS mutagenesis in Arabidopsis.

The high mutation frequencies of the dms4-1 suppressor mutations were reproduced in a second trial of EMS mutagenesis using a more advanced generation of dms4-1 seeds (BC2F3). In a population of 3575 M1 plants and their M2 progeny, we retrieved 17 plants containing one of the four dms4-1 intragenic suppressor mutations identified previously, again giving an average mutation frequency of $\sim 1.2 \times 10^{-3}$ (Table 1).

Mutation frequency is not elevated genome-wide

The high frequencies of dms4-1 intragenic suppressor mutations suggested the existence of a hypermutational process that affected the dms4 gene. To test whether a similar elevated mutation frequency would be observed genome-wide, we carried out Illumina whole-genome sequencing on two independently derived strains from the dms4-1s1 and dms4-1s3 suppressor mutants, respectively, and one strain from the dms4-1s4 suppressor mutant. For comparison, we sequenced the genome of the dms4-1 mutant from the BC1F3 generation, which was used for the first EMS mutagenesis treatment in the suppressor screen (Figure 1A), as well as the wild-type transgenic line containing the GFP target locus and silencer locus (T+S) that was used in the original forward screen that identified the dms4-1 mutant (Kanno et al. 2010).

Once a list of single nucleotide polymorphisms (SNPs) was established for each line, we subtracted SNPs present in the wild-type T+S transgenic line and in the dms4-1 mutant. The remaining SNPs were considered to be ones that were induced by EMS mutagenesis during the suppressor screen. From this analysis, the total number of mutations induced by EMS treatment of dms4-1 seeds ranged from 826 (dms4-1s1, strain 12-1-3) to 2140 (dms4-1s3, strain 3-2-3) (Table 2). These numbers are within the range reported previously...
for EMS mutagenesis in Arabidopsis (Jander et al. 2003) and hence do not support the occurrence of genome-wide hypermutation in the suppressor mutants. Indeed if hypermutation were occurring throughout the genome at the same frequency observed for the dms4-1 gene, then we should have detected ~53,000 mutations in each suppressor mutant [average mutation frequency of dms4-1 suppressor mutations (1.2 × 10⁻³; Table 1) times 4.4 × 10⁷, which is the number of base pairs susceptible to EMS mutagenesis in Arabidopsis (Jander et al. 2003)].

The mutations were distributed throughout the genome and they affected different categories of sequences (e.g., genes, transposons, pseudogenes) at approximately the same percentage at which they are represented in the Arabidopsis genome (Figure 5). The neighboring nucleotides of the G-to-A transitions induced by EMS treatment are consistent with previously reported results (Greene et al. 2003), with purines being favored in the −1 position (Table S4). There was virtually no overlap among the sets of genomewide mutations detected in each suppressor mutant; that is, each mutation was induced independently and only once in the M₁ seed population. This contrasts to the suppressor mutations, which were induced independently multiple times (Table 1). We confirmed a subset of strain-specific SNPs by using cleaved amplified polymorphic sequence (CAPS) markers (Figure S3).

**The dms4-1 allele is not a general target of enhanced mutagenesis**

The four suppressor mutations were straightforward to detect because they led to reversion of dms4-1 mutant phenotypes (that is, the suppressor mutants were GFP⁺ and had a normal developmental phenotype). To determine whether other G residues in the dms4-1 allele were mutated frequently even in the absence of phenotypic reversion, we sequenced the dms4-1 gene in 100 GFP⁺ M₂ plants. No additional mutations were observed in the dms4-1 allele in any of these plants, indicating that the dms4-1 allele as a whole is not a preferential target of mutagenesis.

**Mutations in the GFP reporter gene**

We also determined the frequency of recessive, loss-of-function mutations occurring in the GFP reporter gene. These mutants could be screened out because they were GFP⁻ but had a dms4-1 developmental phenotype. Sixteen independent GFP⁻ mutants resulting from mutations in the GFP coding sequence were obtained in the initial screen of M₂ progeny from ~10,600 M₁ plants resulting from the first EMS treatment. Although most mutations were observed only once, two were observed multiple times. The resulting mutation frequencies (1.9 × 10⁻⁴ and 2.8 × 10⁻⁴, respectively) are somewhat elevated over previously reported average values (Jander et al. 2003) but do not reach the higher frequencies observed with the dms4-1 allele (Figure S4).

**Discussion**

In a screen for genetic suppressors of the dms4-1 mutation, which conditions defects in both RdDM/GFP silencing and plant development, we identified four dominant intragenic suppressor mutations. The suppressor mutations correct all of the dms4-1 mutant phenotypes and hence confirm that both the developmental abnormalities and epigenetic deficiencies observed in the dms4 mutant are due solely to the dms4-1 mutation. The remarkable aspect of this suppressor

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**Table 2 Number of EMS-induced mutations in suppressor mutants**

|     | Chr 1 | Chr 2 | Chr 3 | Chr 4 | Chr 5 | Total |
|-----|-------|-------|-------|-------|-------|-------|
| dms4-1s1 (6-3-5) | 169   | 183   | 219   | 216   | 266   | 1053  |
| dms4-1s1 (12-1-3) | 192   | 123   | 182   | 170   | 159   | 826   |
| dms4-1s3 (3-2-3) | 448   | 331   | 442   | 362   | 557   | 2140  |
| dms4-1s3 (3-4-2) | 295   | 151   | 284   | 143   | 166   | 1039  |
| dms4-1s4 (26-4a) | 242   | 94    | 199   | 160   | 196   | 891   |
| **Average**     | 262.9 | 176.4 | 265.2 | 210.2 | 268.8 | 1189.8 |

Whole-genome sequencing was performed on five suppressor mutants (strain number in parentheses) containing the indicated suppressor mutations (left). The number of G/C to A/T mutations on each chromosome as well as the total number of G/C to A/T changes genome-wide (ranging from 826 to 2140) is shown.
screen, however, was the high frequency with which we re-
covered the suppressor mutations. The average frequency of
the four dms4-1 suppressor mutations was ~100 times
higher than normally observed with standard EMS mutagen-
esis in Arabidopsis. As revealed by whole-genome sequenc-
ing, the mutation frequency was not elevated genome-wide
in the suppressor mutants. Rather, four specific sites in the
dms4-1 allele appeared to be the targets of recurrent muta-
tional events that were recovered at high frequency in our
screen. Whole-genome sequencing confirmed that the four
suppressor mutations were induced independently because
each suppressor mutant contained, in addition to the specific
suppressor mutation, hundreds of additional unique muta-
tions. Moreover, the repeated and independent induction of
the suppressor mutations was illustrated by their presence in
multiple M1 plants as well as M2 progeny grown from sepa-
rate pools of seeds.

Our results are reminiscent of those reported in a pre-
vious study on hypermutation in the bal1 variant in Arabi-
dopsis. The bal1 variant arose during inbreeding of the
epigenetic mutant ddm1, which is defective in a chromatin
remodeler required for DNA methylation of repeated se-
quencies. The bal1 allele conditions a dwarf phenotype,
which is due to constitutive overexpression of the SNC1
gene that is part of a resistance gene cluster. Following
treatment with EMS, the bal1 variant was phenotypically
unstable, with nearly one-third of M1 plants exhibiting
wild-type sectors (Yi and Richards 2008). An epigenetic
source was initially suspected to account for the high fre-
cquency of phenotypic instability. However, subsequent
work demonstrated a genetic mechanism involving duplica-
tion of the SNC1 gene followed by an apparent hypermuta-
tional process that induced inactivating missense mutations
in one copy, thus returning SNC1 expression to a normal
level. Mutagenesis appeared to be restricted to the SNC1
duplication because a sequenced control region lacked
mutations (Yi and Richards 2009).

To explain the high incidence of phenotypic suppression
in bal1 variants, the authors proposed two hypothetical mech-
nisms that are not mutually exclusive (Yi and Richards
2009). The stress-induced mutagenesis hypothesis invoked
DNA damage induced by EMS acting as the stressful agent.
The meristem selection hypothesis proposed that cells car-
ying a mutation in SNC1 outcompete wild-type cells in the
SAM. These hypothetical mechanisms can also be applied to
our results by positing targeted mutagenesis of the dms4-1
allele or selection of revertant cells containing a dms4-1
suppressor mutation in the SAM.

Targeted mutagenesis implies that specific nucleotides
are preferentially mutated at high frequencies. It is not clear
why the four sites sustaining suppressor mutations in the
dms4-1 allele would be preferential targets of mutagenesis.
The wild-type DMS4 gene, which is present as a single copy
in Arabidopsis, is not a preferential target of EMS-induced
mutagenesis in wild-type plants. In the initial screen for dms
mutants in a population of ~52,000 M1 plants, we identified
only two loss-of-function alleles in the DMS4 gene (Kanno
et al. 2010). By contrast, in the current dms4-1 suppressor
screen, the intragenic suppressor mutations were identified
51 and 17 times, respectively, in populations of only ~10,600
and 3575 M1 plants. The nature of the dms4-1 mutation,
which is in a splice-site acceptor of the sixth intron, may
be relevant. Three of four intragenic suppressor mutations
create new splice site acceptors that override the original
dms4-1 mutation, leading to restoration of the wild-type
open reading frame. Analysis of cDNAs in the dms4-1 mut-
ant (Kanno et al. 2010) and the suppressor mutants (this
study) revealed the occurrence of alternative splicing. A
speculative idea is that oscillations between different splic-
ing site acceptors may preferentially fix mutations at spe-
cific splice sites in the dms4-1 allele if they result in a mRNA encoding a functional DMS4 protein. Splicing-
related parameters have been correlated with the selection
of mutations in p53 in human cancers (Koudou et al. 2009).
The somewhat elevated frequency of mutation at two sites
in the GFP reporter may appear to support targeted muta-
genesis at some sites in the genome. Owing to its transgenic
nature, however, it is difficult to judge the relevance of the
results on the GFP reporter gene for mutation frequencies in
endogenous genes.

A common feature of the bal1 variant and the dms4
mutant is that both are likely to harbor widespread epi-
genetic alterations in their genome. As mentioned above,
bal1 arose in the epigenetic mutant ddm1, which accrues
epigenetic and genetic alterations during inbreeding.
DMS4 can be considered an epigenetic factor because it
directly or indirectly affects RdDM (Kanno et al. 2010).
Because active demethylation of DNA is a base-excision
repair process that can potentially be mutagenic (Zhu
2009), the epigenetic state of genes may make them more
or less susceptible to EMS mutagenesis. However, we did not
observe any differences in the DNA methylation state of the
dms4-1 allele compared to that of the wild-type DMS4 gene
(Figure S5).

Perhaps a more plausible explanation than targeted mu-
tagenesis to account for the frequent recovery of the four
suppressor mutations is that they restore DMS4 function and
a normal plant phenotype. DMS4 is important for develop-
ment and dms4-1 suppressor mutations that reestablish
a wild-type phenotype may provide a selective advantage
over mutant cells in the SAM. Such intraorganismal se-
lection is referred to as somatic, cell-lineage or diplontic
selection (Clarke 2011). The young SAM in Arabidopsis
consists of 50–70 cells (Medford 1992) arranged in sev-
eral layers. The L1 layer forms the colorless epidermis, the
L2 layer forms the subepidermis and germ cells, and the
inner L3 region forms core tissues. Cells in L1 and L2 divide
in an anticlinal (sideways) manner, which normally en-
sures that these layers are maintained separately from each
other, whereas cell division in L3 occurs more randomly.
Despite such compartmentalization, occasionally divisions
can take place such that daughter cells invade a different
layer (Carpenter and Coen 1995; Clarke 2011). The stratified SAM of flowering plants would appear to reduce diplontic selection against deleterious mutations (Klekowski 2003) but there is little information on the positive selection of beneficial mutations in the SAM (Carpenter and Coen 1995; Szymkowski and Sussex 1996; Klekowski 2003; McKey et al. 2010).

For diplontic selection to explain our results, a single revertant cell resulting from EMS mutagenesis would have to rapidly outcompete dms4-1 mutant cells and spread to occupy a large portion of the SAM. This presumably takes place during early growth of the M1 seedling because at the adult stage, M1 suppressor mutants appear completely wild-type or contain obvious wild-type sectors. Moreover, the suppressor mutations are consistently inherited in M2 progeny, indicating they were present in the pair of germ cell progenitors in the L2 layer of the SAM of M1 plants (Yi and Richards 2008). The delay in germination and development seen in the dms4-1 mutant at the seedling level (He et al. 2009; Kanno et al. 2010) can possibly be extrapolated to infer a growth advantage of revertant cells over dms4-1 mutant cells in the SAM. In this context, it is interesting to note that nearly 130 cell cycle and cyclin-related genes are differentially regulated in the dms4-1 mutant (Kanno et al. 2010), which might alter cell cycle control in a disadvantageous manner.

The precise mechanism(s) resulting in the frequent and recurrent recovery of dms4-1 suppressor mutations remains to be clarified. However, our work and the previous study on the bal1 variant suggest that Arabidopsis can frequently target or select for beneficial mutations during the lifetime of an individual plant and transmit these mutations to the next generation. The involvement of two different genes (SNCCI and dms4) and two different types of mutations (missense mutations and splice site acceptor mutations) suggest that such phenomena are not restricted to a single system but may actually be quite common in plants. Although our experiments involve EMS mutagenesis of the dms4-1 mutant, they nevertheless illuminate the possibility of positive selection acting on naturally induced genetic variation in the SAM and are consistent with considerable organizational flexibility of the SAM in Arabidopsis (Furner and Pumfrey 1992, 1993) when strong selective forces are at play. The dms4 mutant provides a good system for further analysis of this apparent targeted mutation/selection process and its possible role in plant adaptation and evolution.

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Unusual Case of Apparent Hypermutation in *Arabidopsis thaliana*

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Figure S1  Transgene-based meristem silencing system. In the two component transgene silencing system, a target (T) locus contains a GFP reporter gene downstream of a minimal promoter and an upstream enhancer that drives GFP expression in shoot and root meristem regions (left photo, T). An unlinked silencer locus (S) contains an inverted DNA repeat (heavy black arrows) of distal enhancer sequences (dark shade) that is transcribed by Pol II from the 35S promoter. The resulting hairpin RNA is processed by DCL3 to produce 24-nt siRNAs that induce DNA methylation of distal enhancer sequences through Pol V pathway components, including DRD1, DMS3, DMS4 and AGO4/6. Methylation silences GFP expression (middle photo, T+S). GFP silencing is released in Pol V pathway mutants, including dms4-1 (right photo).
Figure S2  cDNA sequences of DMS4 gene in suppressor mutants. The intron-exon structure of the DMS4 gene is shown at the top. Below is the processed mRNA (spliced introns denoted by peaked lines). Light blue boxes denote UTRs, dark blue boxes coding sequences. The position of the dms4-1 mutation (G to A at the splice site acceptor of the sixth intron) is indicated by the arrow. A conserved domain of the DMS4 protein is delineated by the black bar. Small arrows represent primers used for RT-PCR. Cloned DMS4 cDNAs were sequenced from WT plants, the dms4-1 mutant and the four suppressor mutants: dms4-1s1 to dms4-1s4. Blue and red regions indicate those cDNAs with correct reading frames or incorrect reading frames, respectively (numbers of clones sequenced are shown as denominators to the right; the number with a correct reading frame is shown in bold). Horizontal bars denote unspliced intronic sequences. The gain (+) or loss (-) of nucleotides in mutant cDNAs are shown within unspliced intronic sequences or at the intron-exon junctions.
Figure S3  Genotyping of strain-specific SNPs in dms4-1s1 by cleaved amplified polymorphic sequences (CAPS). To confirm that unique SNPs were indeed restricted to specific strains of suppressor mutants, CAPS markers were designed to detect several selected SNPs specific for dms4-1s1 strain 6-3-5 (635-1 and 635-2) (A) and dms4-1s1 strain 12-1-3 (1213-1 to 3) (B).
Figure S4  EMS-induced mutations in GFP reporter gene. The GFP reporter gene can be used to assess the mutation frequency of a selectively neutral gene. In the first EMS mutagenesis treatment, which involved ~10,600 M₁ plants, we identified 16 GFP-negative M₂ plants that had a dms4-1-like phenotype. In these cases, the GFP-negative phenotype was due to recessive loss-of-function mutations in the GFP gene itself. Although most of the mutations were observed only once (mutation frequency 1/10,600 or 9.4 x 10⁻⁵), two were observed more than once: two times G203A (mutation frequency 2/10,600 or 1.9 x 10⁻⁴) and three times G290A (mutation frequency 3/10,600 or 2.8 x 10⁻⁴). These mutation frequencies are lower than those observed for the dms4-1 suppressor mutations (Table 1).
Figure S5  DNA methylation in the DMS4 gene region around dms4-1 mutation. Bisulfite sequencing was used to examine DNA methylation in the region around the dms4-1 mutation. The DMS4 gene contains CG methylation in the gene body (http://neomorph.salk.edu/epigenome/epigenome.html) and this methylation pattern appeared the same in the wild-type T+S line and the dms4-1 mutant. Vertical bars indicate percent methylation (left) at Cs in CG dinucleotides in the boxed regions of the DMS4 gene.
| primer name     | sequence                                                                 | Purpose                  |
|-----------------|---------------------------------------------------------------------------|--------------------------|
| EPRV_Top2F      | GCG GTG TYA TYT ATG TTA YTA GAT                                        | Bisulfite for target     |
| EPRV_Top2R      | CTT CTT RAT RTT CCA TAR CTT TCC                                        |                          |
| PHV_S-F2        | GGA YYA TAG TGA TGY YAT ATT GTG                                        | Bisulfite for PHV        |
| PHV_S-R         | TAT CAT CAA CAA CTT TCC ACA CC                                        |                          |
| 3028-3          | GAA GCC TGT GAT TGT TAG AG                                              | RT-PCR for DMS4          |
| DMS4sqR2        | CGG TAA TTC TCT TTA GTA TC                                              |                          |
| AtSN1for        | ACC AAC GTG CTG TTG GCC CAG TGG TAA ATC                                | chop-PCR for AtSN1       |
| AtSN1rev        | AAA ATA AGT GGT GGT TGT ACA AGC                                       |                          |
| IGN25for        | CTT CTT ATC GTG TTA CAT TGA GAA CTC TTT CC                              | chop-PCR for IGN25       |
| IGN25rev        | ATT CGT GTG GCC TGG GCC TCT T T                                        |                          |
| 635-1f          | GCG TCT ACC GTT TAG GGC TG                                             | CAPS (Nco I) for 635-1   |
| 634-1r          | GCT TCT TCA GAC CCT CGA GG                                             |                          |
| 635-2f          | CTT TAG GGG TCT CAG TCT CC                                             | CAPS (Nco I) for 635-2   |
| 635-2r          | GTA CAC CGG TAT GAT TCC TC                                             |                          |
| 1213-1f         | GAG CTT TAG GGA AGC TAA TC                                             | CAPS (Nco I) for 1213-1  |
| 1213-1r         | CCG CCA AGA AAC AGT GAC AG                                             |                          |
| 1213-2f         | CTT CAG CCA GTG AGT TGC AC                                             | CAPS (Hind III) for 1213-2|
| 1213-2r         | CAT GTT TCA CCA TCA TCA GTA GC                                       |                          |
| 1213-3f         | CGA TTT CGG AGT CGG AGT CG                                             | CAPS (Xho I) for 1213-3  |
| 1213-3r         | CCG TTG GAG GTA CAC TCT CC                                             |                          |
### Table S2  Suppressor mutants screened from M₂ generation

| strain name | mutation | GFP⁺ | GFP⁻ | total |
|-------------|----------|------|------|-------|
| 2-1a*       | dms4-1s2 | 9    | 9    | 18    |
| 3-1a        | dms4-1s3 | 30   | 7    | 37    |
| 3-2a        | dms4-1s1 | 5    | 19   | 24    |
| 4-2a*       | dms4-1s1 | 15   | 10   | 25    |
| 7-4a        | dms4-1s1 | 16   | 5    | 21    |
| 8-1a*       | dms4-1s3 | 15   | 7    | 22    |
| 9-2b        | dms4-1s2 | 25   | 0    | 25    |
| 16-1a*      | dms4-1s1 | 9    | 11   | 20    |
| 16-1b*      | dms4-1s1 | 16   | 10   | 26    |
| 16-1c*      | dms4-1s1 | 17   | 13   | 30    |
| 18-2a*      | dms4-1s1 | 23   | 13   | 36    |
| 19-3a       | dms4-1s2 | 11   | 9    | 20    |
| 24-1a       | dms4-1s1 | 18   | 0    | 18    |
| 26-4a       | dms4-1s4 | 30   | 6    | 36    |
| 30-2a       | dms4-1s1 | 21   | 4    | 25    |
| 32-2a       | dms4-1s3 | 5    | 20   | 25    |
| 32-3a       | dms4-1s2 | 22   | 13   | 35    |
| 34-1a       | dms4-1s1 | 15   | 10   | 25    |
| 35-3b       | dms4-1s3 | 35   | 11   | 46    |

* Plants in which the dms4 gene was sequenced in the M₁ generation; for the others, the dms4 gene was sequenced in M₂ generation.

Nineteen suppressor mutants containing one of four dominant intragenic suppressor mutations (dms4-1s1 to dms4-1s4) were identified in a population of 10,599 M₁ plants following the first EMS mutagenesis of dms4-1 seeds (BC₁ F₁ generation). Selfed progeny of these plants (M₂ generation) segregated at least some GFP-negative progeny, indicating transmission of the suppressor mutations to the next generation and restoration of GFP silencing. Probably due to chimerism (Fig. 1A-C), segregation ratios of GFP-negative to GFP-positive were not always 3 to 1, as would normally be expected for progeny of an M₁ plant that is heterozygous for a dominant suppressor mutation. Two of the 19 M₁ plants, 9-2b and 24-1a, segregated 100% GFP-negative M₂ progeny. This result is difficult to explain unless the parental plants were homozygous for the suppressor mutation. This may have occurred through an early gene conversion event or if the same intragenic suppressor mutation was induced in both alleles of the dms4-1 gene.
| Batch No. | mutation       | homozygous | heterozygous | screened No. |
|-----------|----------------|------------|--------------|--------------|
| 3-2       | dms4-1s3       | 1          | 3            | 4            |
| 3-4       | dms4-1s3       | 3          | 5            | 8            |
| 4-2       | dms4-1s1       | 2          | 5            | 7            |
| 4-4       | dms4-1s4       | 1          | 0            | 1            |
| 5-3       | dms4-1s2       | 2          | 4            | 6            |
| 5-4       | dms4-1s1       | 1          | 3            | 4            |
| 6-2       | dms4-1s3       | 1          | 10           | 11           |
| 6-3       | dms4-1s1       | 2          | 4            | 6            |
| 7-4       | dms4-1s1       | 0          | 1            | 1            |
| 10-1      | dms4-1s2       | 0          | 5            | 5            |
| 10-3      | dms4-1s4       | 1          | 1            | 2            |
| 12-1      | dms4-1s1       | 1          | 4            | 5            |
| 13-1      | dms4-1s4       | 1          | 2            | 3            |
| 13-4      | dms4-1s1       | 2          | 2            | 4            |
| 14-1      | dms4-1s1       | 3          | 2            | 5            |
| 15-3      | dms4-1s1       | 0          | 2            | 2            |
| 16-1      | dms4-1s2       | 1          | 0            | 1            |
| 16-4      | dms4-1s4       | 3          | 3            | 6            |
| 17-4      | dms4-1s1       | 0          | 2            | 2            |
| 20-4      | dms4-1s1       | 0          | 1            | 1            |
| 23-1      | dms4-1s1       | 2          | 3            | 5            |
| 23-1      | dms4-1s3       | 0          | 1            | 1            |
| 23-2      | dms4-1s2       | 0          | 6            | 6            |
| 23-3      | dms4-1s2       | 0          | 7            | 7            |
| 23-4      | dms4-1s1       | 4          | 2            | 6            |
| 24-2      | dms4-1s1       | 1          | 0            | 1            |
| 24-3      | dms4-1s1       | 2          | 2            | 4            |
| 25-3      | dms4-1s2       | 1          | 8            | 9            |
| 29-3      | dms4-1s1       | 1          | 3            | 4            |
| 32-1      | dms4-1s1       | 0          | 3            | 3            |
| 33-1      | dms4-1s1       | 2          | 0            | 2            |
| 33-3      | dms4-1s4       | 2          | 1            | 3            |

Total 135

Approximately five-seven M$_2$ progeny (actual range 1-11) from each M$_1$ plant (population size 10,599) were germinated on solid MS medium and screened at the seedling stage for a GFP-negative phenotype, indicating restoration of GFP silencing. Thirty-two GFP-negative seedlings were identified. DNA sequence analysis of the dms4-1 gene in these seedlings revealed that they were all homozygous or heterozygous for one of four dominant intragenic suppressor mutations (dms4-1s1 to dms4-1s4). After transfer to soil, the GFP-negative seedlings all lacked features of the dms4-1 developmental phenotype as adult plants.
EMS is an alkylating agent that targets G to produce O\textsuperscript{6}-ethylguanine, which is able to base pair with T but not C. During subsequent DNA repair, the original G/C pair can be replaced by A/T. In the table, zero is the position of the mutated G and the percentages of nucleotides downstream (-5 to -1) and upstream (+1 to +5) observed from whole genome sequencing of suppressor mutants are shown. The neighboring nucleotides of the G to A transitions induced by EMS treatment are consistent with previously reported results, with purines being favored in the -1 position (Greene et al., 2003).

Greene EA et al. (2003) Spectrum of chemically-induced mutations from a large-scale reverse-genetic screen in Arabidopsis. Genetics 164: 731-740.