RESEARCH PAPER

SLTAB2 is the paramutated SULFUREA locus in tomato

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Abstract

The sulfurea (sulf) allele is a silent epigenetic variant of a tomato (Solanum lycopersicum) gene affecting pigment production. It is homozygous lethal but, in a heterozygote sulf/+, the wild-type (wt) allele undergoes silencing so that the plants exhibit chlorotic sectors. This transfer of the silenced state between alleles is termed paramutation and is best characterized in maize. To understand the mechanism of paramutation we mapped SULF to the orthologue SLTAB2 of an Arabidopsis gene that, consistent with the pigment deficiency, is involved in the translation of photosystem I. Paramutation of SLTAB2 is linked to an increase in DNA methylation and the production of small interfering RNAs at its promoter. Virus-induced gene silencing of SLTAB2 phenocopies sulf, consistent with the possibility that siRNAs mediate the paramutation of SULFUREA. Unlike the maize systems, the paramutagenicity of sulf is not, however, associated with repeated sequences at the region of siRNA production or DNA methylation.

Key words: Auxin, DNA methylation, paramutation, photosynthesis, RdDM, siRNA, SULFUREA, VIGS.

Introduction

Paramutation involves the transfer of epigenetic marks from a (paramutagenic) silent gene to the active (paramutable) allele so that it becomes heritably silent and paramutagenic. Several plant species exhibit paramutation and the best characterized examples, the b1 and pl1 loci in maize, have been linked to the process of RNA-directed DNA methylation (RdDM) (Chandler and Stam, 2004; Hollick, 2012) in which paramutagenic small interfering (si)RNAs mediate silencing of the paramutable allele. This simple model does not, however, explain why most siRNA loci are not paramutagenic: there must be other factors.

To shed light on the mechanism of paramutation we are analysing the tomato SULFUREA (SULF) locus. The silent sulf allele has a chlorotic phenotype (Hagemann, 1958) that is associated with reduced auxin (Ehlert et al., 2008). A sulf homozygote is seedling lethal but a viable heterozygous sulf/+ plant has large chlorotic sectors that are due to paramutation of the active allele to a silenced state in early development. This system is like classic maize paramutation because the paramutated state is heritable and paramutagenic (Hagemann, 1969). SULF maps to the pericentromeric heterochromatin of chromosome 2, at approximately 29 cM from the S locus (Soly02g077390, compound inflorescence) (Hagemann and Snoad, 1971) but the affected gene could not be mapped precisely due to low recombination frequency in this region (The Tomato Genome Consortium, 2012).

A gene orthologue of the Arabidopsis ATAB2 is strongly down-regulated in chlorotic sectors of sulf/+ tomato (Ehler...
et al., 2008) but it was previously excluded as the SULF gene because it is still expressed at detectable levels. However, from analysis of transcriptome, methylome, and small RNA populations of wild-type and paramutated tomato leaves we show here that paramutation of SLTAB2 is responsible for the sulf chlorosis and the decrease in auxin levels. SLTAB2 silencing was associated with changes in DNA methylation and siRNA levels at its promoter, a signature of RdDM.

Additional evidence supporting the identification of SLTAB2 as SULF is from virus-induced gene silencing (VIGS) of the SLTAB2 promoter resulting in methylation of the target DNA sequence, silencing of its expression, and a phenocopy of the sulf chlorosis. Together, these results support a causal role of siRNAs and RdDM in paramutation but, unlike the maize examples, the SLTAB2/SULF locus lacked repeated sequences. Mapping of SULF to SLTAB2 and further comparison with maize will help build a general model of paramutation in plants.

Materials and methods

Plant material and growth conditions

Atab2 T-DNA knockouts (GABI-KAT line 354B01) and wild-type Col-0 seedlings were sown on 1/2 strength Murashige-Skoog medium, 1× Nitsch&Nitsch vitamins, 0.8% agar, 1.5% sucrose, pH 6; stratified for 72h at 4 °C in the dark and transferred to short-day conditions (8h light at 23 °C and 50 μmol photons m⁻² s⁻¹, 16h dark at 21 °C). Whole seedlings were collected after 7 d of growth. Tomato plants were raised from seeds in compost (Levington M3) and maintained in a growth room at 23 °C with 16/8h light/dark periods with 60% relative humidity, at a light intensity of 150 μmol photons m⁻² s⁻¹. Young leaves were collected from 1-month-old plants. Sulf and sulf/+ tissue was collected from sulf/+ plants that had both fully yellow (sulf) and fully green (sulf+) sectors.

Transcriptome analysis

Total RNA samples were prepared from 100mg of leaf tissue using TRIZol (LifeTechnologies). For qPCR, 5 μg of total RNA was first DNase treated using Turbo DNase (Ambion), following the manufacturer's guidelines. cDNA was then synthesized using random hexamers and Oligo(dT) and SuperScript III (LifeTechnologies), according to the protocol. qPCR was performed on a Roche LC480 with SYBR in technical triplicates. mRNA abundance was normalized by the geometric mean of two housekeeping genes TIP41 and EXPRESSED (Coker and Davies, 2003). Genotyping of amplifying cDNA was performed by digesting 100ng purified SLTAB2 amplon with Bael (NEB) for 12h at 25 °C in 1× NEB2.1, 100 μg ml⁻¹ BSA, and 20 μM SAM as per the manufacturer’s instructions, and electrophoresis on a 1.5% agarose gel. Strand-specific RNA-Seq libraries for two wild-types and three pairs of sulf and sulf/+ were made and indexed with the ScriptSeq v2 kit (Epiconcept) according to the protocol after RiboZero treatment (Plant leaf, Epicentre) and sequenced as a pool on one lane of HiSeq 2000 100PE. Sequences were trimmed and filtered with Trim Galore! (default parameters), then sequenced on a HiSeq 2500 125PE. Sequences were trimmed and filtered with Trim Galore! (default parameters), then mapped on the Heinz genome SL2.50 and ITAG2.4 gene models using TopHat2 (Kim et al., 2013) (with parameters -r 200 -mate-std-dev 2 -2

Gene Ontology analysis was performed with the goseq package (v1.20.0, Young et al., 2010) using previously published gene ontology annotation (Koenig et al., 2013), normalizing with mRNA length and running with the following parameters: method=Wallerius, repfilt=2,000, use_genes_without_cat=F. Categories were considered to be over-represented if the associated P-value was <0.05 after Benjamini-Hochberg correction.

Methylome analysis

DNA was extracted from 100mg of leaf tissue (from the same sampling as for RNA-Seq and sRNA-Seq, for two wild-types and two sulf) using the Puregene kit (QIAGEN). Bisulfite library preparation was performed with a custom protocol similar to Urich et al. (2015). 1.2 μg DNA was sonicated on a Covaris E220 to a target size of 400 bp and purified on XP beads (Ampure, ratio 1.8). DNA was size-selected and A-tailed using T4 DNA polymerase and Klenow Fragment (NEB) and purified again using XP beads (ratio 1.8×). Methylated Illumina Y-shaped adapters for paired-end sequencing were ligated using Quick-Step Ligase (Bioline). 450ng of purified (ratio 1.8×), adapter-ligated DNA was bisulfite-converted using the EZ DNA Methylation-Gold Kit (Zymo Research) according to the manufacturer’s instructions. DNA was barcoded using 12 cycles of PCR amplification with KAPA HiFi HotStart Ural+Ready Mix (Kapa Biosoys) with PE1.0 and custom index primers (courtesy of the Sangor Institute). Pooled libraries were sequenced to a depth of about 5× on a HiSeq 2500 125PE. Sequences were trimmed and filtered with Trim Galore! (default parameters), then mapped on the Heinz genome SL2.50 using Bismark v0.14.3 (Krueger and Andrews, 2011) (first in paired-end mode with options --score-min 50, --mate-gap 20 --mate-max 400 -2

Bismark methylation extractor (with options -r 2 for paired end reads). Methylated and unmethylated counts for cytosines of both strands were pooled into contiguous 200bp bins and separated by context (CG, CHG, and CHH) with a custom python script. Bins with fewer than 10 counts were excluded from the analysis. Bins are considered differentially methylated if the maximum P-value of the two chi-square tests (wt1 versus sulf 1, wt2 versus sulf 2) is <0.05. Analysis of methylation by McrBC was performed as previously described by Bond and Baulcombe (2015). For Sanger bisulfite sequencing, 450ng of DNA was bisulfite-converted, amplified with primers specific to the region of interest, A-tailed, and cloned into pGEM-T easy (Promega) following protocols similar to the library

sRNA-Seq

sRNAs were cloned from 10 μg total RNA (from the same tissue as used for RNA-Seq, for two wild-types and two pairs of sulf and sulf+/+) using the Illumina TruSeq Small RNA cloning kit and libraries were indexed during the PCR step (12 cycles) according to the manufacturer's protocol. Gel size-selected, pooled libraries were sequenced on a HiSeq 2000 50SE. Sequences were trimmed and filtered with Trim Galore! (with the adapter parameter -a TGGAATTCTCGGGTGCCAAGG) and 14–20 million reads per library were mapped without mismatches and clustered on Heinz genome SL2.50 using the ShortStack software v2.1.0 (Axtell, 2013; Supplementary Table S2). sRNA counts on the defined loci were analyzed with DESeq2 v1.8.1. Uniquely mapping reads on DMR1 and DMR2 were normalized with edger’s implementation of TMM size factors, on all sRNAs present in all libraries and with at least 10 total counts (Robinson et al., 2009), and a Poisson regression was applied to the normalized counts (generalized linear model in R, with the genotype variable taking values wt, sulf,Idle, and sulf).


preparation. Sequences aligned with MUSCLE were then analysed with CyMATE (Hetzl et al., 2007).

**VIGS**

DMR1a (606 bp) and DMR1b (562 bp) genomic inserts were cloned into the binary TRV RNA2 vector using the KpnI and XhoI restriction sites of the multiple cloning site as described previously (Liu et al., 2002; Bond and Baulcombe, 2015). Cotyledons of tomato seedlings were agro-infiltrated 10 d after sowing with a 1:1 mixture of Agrobacterium tumefaciens (strain GV3101:pMP90+pSOUP) carrying TRV RNA1 and RNA2 at OD_{600}=1.5. Symptoms of SLTAB2 silencing were visible from 2 weeks post-infection.

**Auxin quantification**

Endogenous levels of free IAA were detected by LC-MS/MS method as described in Novák et al. (2012). Briefly, 10–20 mg fresh tissue of the control and mutant lines were collected, extracted in ice-cold 50 mM sodium phosphate buffer (pH 7) and purified by SPE on hydrophilic–lipophilic balance reversed-phase sorbent columns (Oasis HLB, 1 cc/30 mg, Waters). To each extract, 5 pmol of 13C6-IAA were added as internal standards to validate the quantification. Purified samples were analysed by the LC-MS/MS system consisting of an ACQUITY UPLC System (Waters, Milford, MA, USA) and Xevo TQ-S (Waters) triple quadrupole mass spectrometer. Quantification was obtained using a multiple reaction monitoring (MRM) mode of selected precursor ions and the appropriate product ions.

**Oligonucleotides**

Please refer to Supplementary Table S4.

**Accession codes**

All sequencing data have been deposited in the Sequence Read Archive under the BioProject SRP066362.

**Results**

**Pericentromeric SLTAB2 is strongly down-regulated in sulfurea**

The tomato lines in this study had either unsilenced SULF loci (wild type) or they were the progeny of a cross between the wild type and a plant with sectors with silent soy. Some of the F1 plants were wild type and fully green or, like the sectored parent, they had green and chlorotic sectors consistent with a heterozygous sulf+/+ epigenotype with paramutation. The chlorotic sectors would have had the homozygous sulf/sulf epigenotype (referred to as sulf) and the non-paramutated green sectors would be sulf+/+ (Fig. 1A).

Based on the understanding of paramutation in maize, the SULF locus would be suppressed in sulf (paramutated yellow sectors), partially silent in sulf+/+ (non-paramutated green sectors) and fully expressed in the wild type (wt). It would also be located in the pericentromeric heterochromatin of chromosome 2 upstream of the euchromatic S locus (Hagemann and Snoad, 1971). To find loci with these characteristics, we analysed transcripts of wild-type, non-paramutated sulf+/+, and paramutated sulf leaves using mRNA-Seq. We identified 2237 differentially expressed genes between sulf and wt (P <0.05) that clustered into four main categories with distinct Gene Ontology enrichments (Fig. 1B; Table 1). Consistent with a decrease in photosystem I and the quantity of pigment in sulf leaves (Ehlert et al., 2008), many photosynthesis-related genes were down-regulated (Table 1, class II). The down-regulation of photosystem I was also detectable in the non-paramutated heterozygous sulf+/ leaves (Table 1, class I). Genes associated with various stress responses were up-regulated in sulf (Table 1, class IV) and were probably a secondary consequence of the sulf phenotype.

Of these differentially expressed genes, 36 were both down-regulated in sulf and located upstream of the S locus on chromosome 2. Among these candidates for SULF, Solyc02g005200 particularly stood out as being the most repressed in sulf (15-fold reduction) and at the predicted map position of SULF (29 cM from S locus, when the centromere–S distance is 30 cM). The other candidates mapped to the euchromatin or the transition zone between heterochromatin and euchromatin (Fig. 1C; Supplementary Table S5). Further qPCR analysis confirmed the strong down-regulation of Solyc02g005200 in sulf (26-fold) and revealed variable levels in sulf+/+, compatible with mono-allelic expression (Fig. 1D).

Solyc02g005200 is the orthologue of Arabidopsis thaliana ATAB2 that is likely involved in the translation of mRNAs for both photosystems (Barneche et al., 2006) and we refer to it as SLTAB2.

To confirm the heritability of the SLTAB2 silent epiallele, we analysed SLTAB2 in the F1 progeny of chlorotic sulf+/ (S. lycopersicum cv. Lukullus) crossed with S. pimpinellifolium. Out of 22 F1 plants, 8 displayed a paramutated phenotype with yellowing of parts of the leaves (Fig. 2A). The expression of SLTAB2 in these chlorotic plants was reduced by about half in their green sectors compared to wild-type plants, and 9-fold in their yellow sectors (Fig. 2B). Furthermore, in a PCR test that differentiated the polymorphic alleles from the two parents, we only detected expression from S. pimpinellifolium (Fig. 2C). These data are consistent with SLTAB2 being SULFUREA: in the green tissue the S. pimpinellifolium allele would have been expressed (but not the silent allele from the sulf+/ S. lycopersicum parent), and in the chlorotic tissue, it would have been paramutated. In addition, by confirming the heritable silencing of the S. lycopersicum allele in the chlorotic plants, these data confirm that SLTAB2 silencing is not merely a consequence of the sulf phenotype.

Furthermore, consistent with equivalence of SULF and SLTAB2, the Arabidopsis T-DNA knockout of ATAB2 is seedling-lethal in heterotrophic conditions and deficient in green pigment (Barneche et al., 2006; Fig. 3A). This mutant also has less auxin than the wild type (Fig. 3B). These three phenotypes all resemble sulf.

**Paramutation is associated with changes in SLTAB2 promoter DNA methylation**

We predicted, based on the analysis of the maize h1 gene (Stam et al., 2002), that the DNA of the paramutagenic sulf would be hypermethylated. After a genome-wide analysis of differentially methylated regions (DMRs) between wt and
sulf, we looked for candidate loci in the appropriate region of chromosome 2 and adjacent to genes that were differentially expressed. Genome-wide there were thousands of such DMRs in the CHH context and hundreds in the CG and CHG contexts, with the CHH DMRs being predominantly hypermethylated in sulf whereas CG and CHG DMRs were evenly split between hyper- and hypo-DMRs (Fig. 4A). On chromosome 2 there were several differentially expressed genes with adjacent CHH DMRs but only the SLTAB2 locus had strong DMRs in all cytosine contexts (Fig. 4B).

Closer inspection revealed that there are two adjacent DMRs in the immediate promoter of SLTAB2, DMR1
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and DMR2 (Fig. 4C; Table 2). DMR2 overlaps annotated repeats directly upstream of SLTAB2 and was hypomethylated in sulf in the CHG and CHH contexts (78% and 4% methylation, respectively, compared with 93% and 27% in the wild type). DMR1, by contrast, overlaps the transcriptional start site of SLTAB2 from 300 bp upstream, encompassing the first two exons and introns, and it was hypermethylated in sulf in all contexts (73% mCG, 62% mCHG, and 4% mCHH compared with 24%, 4%, and 0.7%, respectively, in wt). This hypermethylation of the transcriptional start site of SLTAB2 is consistent with a decrease in transcription in sulf and it further strengthens the case that SLTAB2 is SULF.

Additional evidence for the relevance of DMR1 methylation for paramutation is our finding that the silencing of the S. pimpinellifolium allele in the chlorotic F1 (sulf+/S. pimpinellifolium) coincided with a hypermethylation of this region (Fig. 4D). The silent S. lycopersicum allele was methylated in all parts of the paramutated plants, whereas the S. pimpinellifolium allele was unmethylated in green sectors and methylated in yellow sectors.

We also predicted, based on the maize paramutation examples, that sulf would correlate with 24-nt siRNAs. At a genome-wide scale, there was a distinct increase in 23–24-nt siRNAs in sulf compared with the wild type (Fig. 5A), in line with the pattern of CHH hypermethylation. At DMR1 of SLTAB2, the 23–24-nt siRNAs were more abundant in paramutated sulf (Fig. 5B) than in the wild type whereas at DMR2 the 23–24-nt siRNAs were less abundant than in the wild type (Fig. 5C).

VIGS of SLTAB2 phenocopies sulf

To test the involvement of SLTAB2 in sulf paramutation further, we used virus-induced gene silencing (VIGS). VIGS, when targeted to transcribed sequences, leads to knock-down of mRNA levels by post-transcriptional gene silencing but, when targeted at DNA sequences [e.g. the FWA promoter in A. thaliana (Bond and Baulcombe, 2015)], it can initiate heritable DNA methylation and transcriptional gene silencing. We cloned two segments (a and b, Fig. 4C) of DMR1 into tobacco rattle virus (TRV)
RNA2 and inoculated them with TRV RNA1 to wild-type tomato. While infection with TRV-DMR1a caused only mild variegation of the leaves, infection with TRV-DMR1b caused almost all plants to develop large sulf-like chlorotic sectors (Fig. 6A). The similarity of this VIGS phenotype to leaves of sulf is further evidence that SLTAB2 and SULF are equivalent.

DNA methylation analysis of chlorotic sectors by McrBC suggested that there is an epigenetic component to the silencing of SLTAB2 by TRV-DMR1b: the targeted DNA was as strongly methylated as in sulf samples (Fig. 6C). The involvement of epigenetics is further supported by the lasting VIGS phenotype several months post-inoculation (Fig. 6B) during which time the level of the virus vector decreased. From these data we conclude that the DMR1b region of SLTAB2 has the predicted characteristics of the paramutagenic component of sulf because it is susceptible to epigenetic modification.

**Discussion**

In this paper we present several lines of evidence that SLTAB2 is SULF: it maps closer to SULF than any other genes with the predicted pattern of mRNA accumulation in sulf/+ and sulf tissue (Fig. 1B); it encodes a protein required for photosystem I production that explains the chlorotic phenotype; the orthologous atab2 mutation has the same chlorosis and auxin-deficient phenotype as sulf (Fig. 3); and there is a definite DNA methylation mark at the silent SLTAB2 allele in sulf that is inherited from sulf/+ both in selfed and outcrossed progeny (Fig. 2). This epigenetic mark...
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is transferred to the previously active allele in F1 progeny (Fig. 4D) and is associated with 24-nt siRNAs (Fig. 5). The final evidence for the equivalence of SLTAB2 and SULF is from the finding that VIGS targeted to the SLTAB2 DMR can recapitulate both the physiological and epigenetic features of sulf (Fig. 6).
We envision that paramutation occurs when methylation of DMR1 DNA by paramutagenic siRNAs starts a positive feedback loop in which Pol IV is recruited to the silent locus by SHH1 (Law et al., 2013). The recruited Pol IV (Blevins et al., 2015; Zhai et al., 2015) would transcribe siRNAs that would mediate maintenance of the silent state of SLTAB2 and its transfer to paramutable alleles.

SLTAB2 had been ruled out previously as SULF because it is expressed at detectable levels in sulf and it was thought that, unlike tomato, the mutation of the Arabidopsis orthologue could be rescued on sucrose (Ehlert et al., 2008). An alternative candidate for SULF was implicated in the tryptophan-independent pathway of auxin biosynthesis. With our new data, however, we show that the previous exclusion of SLTAB2 was not valid because targeted suppression by VIGS or mutation of this gene produces an accurate phenocopy of the sulf phenotype including, in the atab2 mutant, an auxin defect and seedling lethality.

A likely explanation for the sulf phenotype based on silencing of SLTAB2 invokes the failure to translate the psaB mRNA as described for the orthologous mutations ATAB2 in Arabidopsis and TAB2 in Chlamydomonas (Dauvillée et al., 2003; Barneche et al., 2003).

### Table 2. SLTAB2 DMR methylation

| DMR  | Context | wt  | sulf  | P-value |
|------|---------|-----|-------|---------|
| DMR1 | CG      | 21.4; 27.3 | 71.4; 76.8 | <2 × 10⁻¹⁶ |
|      | CHG     | 2.3; 5.8  | 61.9; 62.4 | <2 × 10⁻¹⁶ |
|      | CHH     | 0.5; 0.8  | 3.8; 4.8  | 1.12 × 10⁻⁷  |
| DMR2 | CG      | 95.6; 96.4 | 96.6; 93.7 | 0.557 |
|      | CHG     | 94.4; 90.9 | 78.0; 78.4 | 7.34 × 10⁻⁴  |
|      | CHH     | 25.6; 29.3 | 5.5; 3.6 | <2 × 10⁻¹⁶ |

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**Fig. 5.** sRNAs in sulfurea. (A) MA plot of 24-nt sRNA loci in wt and sulf. Of the differential sRNA loci between wt and sulf (in red, adjusted P < 0.05), 498 had more abundant sRNAs in sulf, while only 48 had more abundant sRNAs in the wild type. (B) sRNA counts on DMR1 in the wild type, heterozygous sulf/+, and homozygous paramutated sulf leaves. 23–24-nt sRNAs are rare but more abundant in sulf (P = 0.0146, Poisson regression). (C) sRNA counts on DMR2. 23–24-nt siRNAs are reduced in sulf (P = 5.48e – 05, Poisson regression). Counts in (B) and (C) are for normalized, uniquely mapping reads.

**Fig. 6.** VIGS of SLTAB2 DMR1. (A) At 3 weeks post-infection, plants infected with TRV-DMR1b displayed large chlorotic sectors. Between 13 and 16 plants were infected for each condition. (B) These sulfurea-like sectors remained in later leaves at 2 months after infection. (C) Methylation of DMR1 is detectable in chlorotic sectors. The proportion of methylation is calculated from the ratio of amplicons in the McrBC-digested versus undigested samples (as determined by qPCR). If all alleles are highly methylated (100% methylation), they will be digested by McrBC and no amplification will occur during qPCR.
The PsbB protein is the reaction centre protein of photosystem I and, in its absence, the thylakoid membranes would fail to form, pigments would not accumulate at the normal levels, and the leaves would be chlorotic. An auxin defect of *sulf* is a likely consequence of the PsbB defect, as observed in the *atab2* mutant (Fig. 3). In addition, the genome-wide hypermethylation in the CHH context in *sulf* is reminiscent of transient hypermethylation in response to stress, already described in phosphate-starved rice (Secco et al., 2015) and virus-infected *Arabidopsis* (Bond and Baulcombe, 2015).

Several cases of paramutation have been tied to the hypermethylation of regulatory tandem repeats (Stam et al., 2002; Chandler and Stam, 2004). By contrast, the *sulfurea* paramutation is correlated with increased DNA methylation at the transcriptional start site (DMR1) where there is no repeated DNA (Fig. 4). There are annotated LTR fragments at DMR2 that is directly adjacent to DMR1 but, unlike the classic systems, this region of the paramutagenic *sulf* allele showed a reduction in sRNAs and hypomethylation. Hypomethylation of DMR2 may be a consequence of a different chromatin state of the silenced allele and, although its repeats may not be directly involved in the silencing of *SLTAB2*, they and the largely heterochromatic region in which *SLTAB2* is embedded may contribute to its paramutability, poising it for silencing.

The opportunity to study paramutation via *SLTAB2/SULF* in tomato has several advantages over the various maize systems that have been most informative until now. First we have a VIGS system so that establishment of the epigenetic mark can be tracked directly in tomato mutants that are defective for components of the RNA silencing pathways. We will also be able to use VIGS on *sulf/+* plants to test the role of various tomato genes in the establishment and maintenance of paramutagenicity and paramutability.

A second benefit of the *sulf* system is the possibility of studying the transfer of the epigenetic mark in vegetative tissue. With the well-studied maize paramutation systems this transfer is likely to occur early in embryo development and is not readily accessible to molecular analysis, whereas, in tomato, it will be taking place in or close to vegetative meristems. It will still not be easy to access the cells in which the allelic transfer is taking place but we may be able to use the DMR1-specific siRNAs as markers of the transfer process. These RNAs are rare in total plant extracts (Fig. 5) but they may be more abundant at the primary sites of paramutation. Having identified the *SULF* gene, we should also be able to complement the physiological consequences by providing a transgene without the target DNA of paramutation so that we can grow plants with a *sulf/sulf* epigenotype.

These various experimental tools will allow us to explore the differences of the tomato and maize paramutation systems. For example, the apparent target of paramutation in *sulf* has no tandem repeats and is close to the transcriptional start whereas, in maize, at the B locus, they are essential and separated from the transcribed region by 100 kb. Answers to these and other questions will allow us to explore the frequency of paramutation-like events in plant breeding and evolution. Several recent findings indicate that such events are not restricted to the few well-characterized examples of paramutation in maize and other species (Regulski et al., 2013; Greaves et al., 2014). They may be frequent and have an effect on transgressive and heterotic phenotypes.
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