REPORT

Chemical enhancers of posttranscriptional gene silencing in *Arabidopsis*

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ABSTRACT

RNAi mediated by small-interfering RNAs (siRNAs) operates via transcriptional (TGS) and posttranscriptional gene silencing (PTGS). In *Arabidopsis thaliana*, TGS relies on DICER-LIKE-3 (DCL3)-dependent 24-nt siRNAs loaded into AGO4-clade ARGONAUTE effector proteins. PTGS operates via DCL4-dependent 21-nt siRNAs loaded into AGO1-clade proteins. We set up and validated a medium-throughput, semi-automatized procedure enabling chemical screening, in a 96-well in vitro format, of *Arabidopsis* transgenic seedlings expressing an inverted-repeat construct from the phloem companion cells. The ensuing quantitative PTGS phenotype was exploited to identify molecules, which, upon topical application, either inhibit or enhance siRNA biogenesis/activities. The vast majority of identified modifiers were enhancers, among which Sortin1, Isoxazolone, and [5-(3,4-dichlorophenyl)furan-2-yl]-piperidine-1-ylmethanethione (DFPM) provided the most robust and consistent results, including upon their application onto soil-grown plants in which their effect was nonautonomous and long lasting. The three molecules increased the RNAi potency of the inverted-repeat construct, in large part by enhancing 21-nt siRNA accumulation and loading into AGO1, and concomitantly reducing AGO4 and DCL3 levels in planta. A similar, albeit not identical effect, was observed on 22-nt siRNAs produced from a naturally occurring inverted-repeat locus, demonstrating that the molecules also enhance endogenous PTGS. In standardized assays conducted in seedling extracts, the three enhancers selectively increased DCL4-mediated processing of in vitro-synthesized double-stranded RNAs, indicating the targeting of a hitherto unknown PTGS component probably independent of the DCL4-cofactor DOUBLE-STRANDED RNA-BINDING 4 (DRB4). This study establishes the proof-of-concept that RNAi efficacy can be modulated by chemicals in a whole organism. Their potential applications and the associated future research are discussed.

Keywords: *Arabidopsis*; chemical screen; enhancers-suppressors; in vitro assay; RNAi; siRNA

INTRODUCTION

In plants, endogenous RNA interference (RNAi) is triggered by the conversion of various sources of long double-stranded (ds)RNAs into populations of small-interfering RNAs (siRNAs). dsRNAs may be spawned by read-through transcription at sense-antisense loci, by the action of RNA-dependent RNA polymerases (RDRs), or by transcribed loci arranged into inverted-repeats (IRs). At the core of siRNA biogenesis is a class of RNase III enzymes called Dicer-like (DCL) proteins, of which there are four representatives (DCL1-4) in the model plant species *Arabidopsis thaliana*. siRNAs are then incorporated into multiprotein effector complexes known as RNA-induced silencing complexes (RISCs) that invariably contain one representative of the conserved ARGONAUTE (AGO) protein family, of which

there are 10 paralogs in *Arabidopsis* (AGO1-10). As part of RISCs, small RNAs (sRNAs) guide AGOs to sequence-complementary DNA or RNA, which they silence via transcriptional (TGS) or posttranscriptional gene silencing (PTGS), respectively (Bologna and Voinnet 2014).

RNAi operated through TGS depends on DCL3-dependent 24-nt siRNAs loaded into AGO4-clade AGOs. The 24-nt siRNAs are mostly spawned by repeats and transposable elements, replete in plant genomes, and mediate RNA-directed DNA methylation (RdDM) primarily at their loci-of-origin, often resulting in TGS when promoter regions are targeted (Cuerda-Gil and Slotkin 2016). Thus, by dampening transcription of TEs, RdDM and TGS contribute to

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the maintenance of genome integrity. RNAi operated via PTGS, on the other hand, functions mainly via DICER-LIKE-4 (DCL4)-dependent 21-nt siRNAs, which, loaded into AGO1-clade proteins, mediate endonucleolytic cleavage or “slicing” of target mRNAs. Twenty-one-nucleotide endo-siRNAs are generated from RDR6-dependent long dsRNA or processed directly from the products of *IR* loci including prototypical *MIRNA* genes (Bologna and Voinnet 2014). Endogenous PTGS plays developmental roles, notably by enabling patterning of leaf polarity and juvenile-to-adult phase transition in *Arabidopsis* and other species via RDR6-dependent, so-called trans-acting (tasi)RNAs (Gasparoli et al. 2005; Poethig et al. 2006). Like other endogenous siRNAs, 21-nt tasiRNAs have non-cell-autonomous effects, yielding PTGS phenotypes that generate activity gradients from their sites of biogenesis, potentially enabling a morphogenetic output (Chitwood et al. 2009; Schwab et al. 2009). Other tasiRNA-like molecules serve to regulate large gene families including *NBS-LRRs* that confer immunity to plants against a variety of pathogens (Shivaprasad et al. 2012; Boccardo et al. 2014). DCL4 and its surrogate, DCL2, play direct antiviral roles by processing dsRNA features of replicating viral genomes into virus-derived siRNAs (vsiRNAs), which, loaded into mainly AGO1 and AGO2, target viral RNA for destruction (Szittyá and Burguán 2013).

A large class of endogenous DCL1-dependent products called microRNAs (miRNAs) derive as single species, as opposed to populations, from imperfect stem-loop precursor transcripts encoded in the genome by dedicated *MIRNA* genes. miRNA biogenesis is a stepwise, DCL1-dependent nuclear process whereby primary miRNA transcripts (pri-miRNAs) are first converted into miRNA stem-loop precursors (pre-miRNAs) from which the mature miRNA is excised, loaded, and exported to the cytosol as a miRNA-AGO complex in a CRM1-dependent manner (Bologna et al. 2018). The DCL1 pri-to-pre-miRNA converting activity is also probably required to excise the double- from single-stranded parts of *IR* transcripts generating the siRNA populations evoked above. Like most PTGS-related siRNAs, miRNAs are loaded nearly exclusively into AGO1-clade AGOs to regulate the steady-state levels (via slicing) and/or rates of translation of target mRNAs displaying perfect or near-perfect sequence complementarity. Also like all plant siRNAs, miRNAs are protected at their 3' ends by 2'-O-methylation mediated by the RNA methyl-transferase HUA ENHANCER 1 (HEN1), the lack of which results in 3' end poly-uridylation and subsequent exonucleolytic degradation of si/miRNA strands (reviewed in Bologna and Voinnet 2014).

Forward and reverse genetics has identified a plethora of DCL1 cofactors required for its nuclear stepwise activities, uncovering highly sophisticated regulatory mechanisms. In contrast, cofactors of the siRNA-producing DCL2, DCL3, and DCL4 remain essentially unknown with the ex-

ception of double-stranded RNA binding (DRB) proteins that interact with and assist some of these enzymes by anchoring their long dsRNA substrates. These DRBs were identified by reverse genetics due to their similarities with DRB1, also known as HYPONASTIC LEAVES 1 (HYL), a key interactor of DCL1 in miRNA biogenesis (Vazquez et al. 2004; Hiraguri et al. 2005; Adenot et al. 2006). In particular, DRB4 physically interacts with DCL4 to facilitate processing of long dsRNA (Adenot et al. 2006), an assisting role negatively regulated by the related DRB4-interacting proteins DRB7.1 and DRB7.2 (Tschoopp et al. 2017). DRBs aside, regulatory mechanisms for DCL2, DCL3, and DCL4 activities, should they exist, remain mysterious, raising the issue of their potential genetic redundancy. Indeed, all siRNA-generating DCLs in *Arabidopsis* share a continuum of hierarchical activities on the same long dsRNA substrates (Deleris et al. 2006). There is also the unexplored possibility that some DCL cofactors may have other RNA silencing-unrelated functions essential for fertility or embryonic growth. Also, null mutations in AGO1, the shared effector of endogenous siRNA-, antiviral- and miRNA-mediated PTGS, cause compromised growth and sterility (Bohmer et al. 1998).

We reasoned that, perhaps, factors facilitating or modulating siRNA biogenesis/activities in *Arabidopsis* might be amenable to chemical compound screens overcoming their potential genetic redundancy, embryonic lethality, and/or sterility. Forward chemical compound screens are commonly used in unicellular organisms and cell cultures. Owing to its small size and well-controlled growth, *Arabidopsis*, as a whole organism, is also amenable to these approaches under appropriate automation and imaging conditions. Chemical screens in *Arabidopsis* have, for instance, enabled studies of hormone signaling, biotic/abiotic stress or gravity sensing (Hicks and Robert 2014). To screen for RNA silencing activities entails, of course, accessible readouts of the underlying molecular mechanisms, which are commonly provided by transgenic activity sensors. While sensor-based chemical screens have revealed likely genetically inaccessible facets of TGS in *Arabidopsis* (Zhang et al. 2012; Dinh et al. 2014), chemical modifiers of PTGS are yet to be documented in any plant species. Identifying PTGS-modifying compounds would not only provide enabling tools to explore the underlying mechanisms, but also, potentially, to modulate traits endowed by experimental RNAi or endo-siRNA-mediated regulations, as well as antiviral defense.

RESULTS AND DISCUSSION

An in vitro chemical screen to identify both inhibitors and enhancers of the *Arabidopsis* PTGS pathway

To evaluate the feasibility of a screen for chemical modifiers of the PTGS pathway, we used the *Arabidopsis* *SUC-SUL* reporter line, in which the phloem companion-cell specific

AtSUC2 promoter drives expression of an *IR* transgene, the key genetic features of which are summarized below. *SUC-SUL* silences non-cell-autonomously the endogenous and ubiquitously expressed *SULFUR* (*SUL*) mRNA, which is involved in chlorophyll biosynthesis. In leaves, this causes a vasculature-centered bleached phenotype (Fig. 1A), which, under controlled in vitro growth conditions manifests itself most robustly at the seedling stage (Leaf 1-to-7). Of the 21-nt (DCL4-dependent) and 24-nt (DCL3-dependent) siRNA species produced from the *SUC-SUL* locus, the former is necessary and sufficient to silence

SUL via PTGS in an AGO1-dependent manner (Dunoyer et al. 2005; Brodersen et al. 2008). Additionally, *SUC-SUL* long dsRNAs over-accumulate in the hypomorphic *dcl1-9* mutant background when the activities of DCL2, DCL3, and DCL4 are simultaneously abrogated, whereas DCL1 sole contribution to *SUL* siRNA production is modest (Dunoyer et al. 2007). As also seen with endo-IRs (Henderson et al. 2006), this suggests that the DCL1 pri-to-pre-miRNA converting activity is required upstream of DCL4 and DCL3 to facilitate dsRNA excision from the single-stranded regions of the *SUC-SUL* primary transcript (Dunoyer et al. 2007). Finally, and counterintuitively given its DCL4-assisting role, bleaching in the *SUC-SUL* system remains unaffected in the loss-of-function *drb4-1* background. The 21-nt siRNA levels are reduced in *drb4-1* yet those of the 24-nt siRNAs are concomitantly strongly increased due, presumably, to DRB3, which assists DCL3, accessing more *SUC-SUL* dsRNA substrates. The excess in 24-nt siRNAs is such that some of these species anomalously mediate PTGS by loading into AGO1 instead of cognate AGO4-clade AGOs (Dunoyer et al. 2007; Haas et al. 2008).

Forward EMS screens conducted previously on *SUC-SUL* have identified genetic modifiers indicating that variations in 21-nt *SUL* siRNA production/movement readily translate into reduced/increased chlorosis (Dunoyer et al. 2005). Thus, *SUC-SUL* could enable isolation of both PTGS-enhancers and PTGS-suppressors via chemical screening. To test this idea, 7-d-old *SUC-SUL* seedlings were grown in vitro into 96-well plates (four-five seedlings per well) under conditions of synchronous development. To ensure high reproducibility, automated liquid-handling robotics was used to spray the 96-well plates with tested chemical compounds (Fig. 1B). Seven days later, each well was inspected digitally under the stereo-microscope for subsequent software-based quantification of chlorosis. The bleached surface was integrated and compared to that of mock-treated seedlings used as a reference, resulting in a percentage of normalized chlorosis. A standard analysis typically involved $N=12$ well

FIGURE 1. Screening and validation of chemical molecules affecting the PTS pathway. (A) Phenotype of a *SUC-SUL* plantlet grown in vitro. (B) Experimental layout to screen and select in vitro chemical molecules that inhibit or enhance the *Arabidopsis* PTGS pathway. (C) Percentage of the chlorotic phenotype of *SUC-SUL* seedlings treated with Sortin1, SAHA, or TSA, normalized to the mock treatment. Error bars, mean $\pm$ 95% confidence intervals; $N=8$. (D) Chlorotic phenotypes of the *SUC-SUL* seedlings treated with mock, Sortin1, SAHA, or TSA. (E) Northern blot analysis of the *SUL* siRNAs in *SUC-SUL* seedlings treated with mock, Sortin1, SAHA, or TSA, as obtained in two independent experiments. (Upper panels) Experiment #1; (bottom panels) experiment #2. (U6) Control for equal RNA loading. Relative quantification of the *SUL* siRNA signals normalized to U6 are indicated below the panels, with 1.00 arbitrary set for mock treatments.

replicates in 96-well plates. Candidate molecules were classified as putative enhancers or putative inhibitors if they respectively increased or reduced the chlorotic surface of *SUC-SUL* seedlings (Fig. 1B).

We anticipated two major sources of false-positives that would possibly confound the screen's readout. The first one would result from molecules that enhance or reduce the activity of the companion cell-specific *AtSUC2* promoter driving the *SUC-SUL* IR construct. These would alter dsRNA production and, consequently, siRNA processing, causing modified bleaching phenotypes not reflecting modified RNAi per se. The second nonmutually exclusive limitation was that of mere phytotoxicity of the molecules, usually manifested as extended chlorotic patches. To circumvent both of these potential caveats, the tested molecules were systematically sprayed, in parallel, onto seedlings of *AtSUC2-GFP* plants (Imlau et al. 1999) without the *SUC-SUL* background and further inspected under normal light (phytotoxicity) and epifluorescence (modified *AtSUC2-GFP* activity) (Fig. 1B).

In vitro validation of the experimental layout using a set of "educated guess" molecules

The practicability/robustness of the above setting was first tested with an 80-molecules panel chosen via educated guess based on our genetic understanding of *SUL* silencing and of PTGS in general. For instance, several EMS-induced mutations inhibiting *SUL* silencing affect genes involved in RdDM and heterochromatin formation, which are also required in related settings of non-cell-autonomous RNAi and suggest the contribution of as yet poorly understood epigenetic components to this process (Dunoyer et al. 2007). Chlorotic surface-measurements showed that suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA), two histone deacetylase inhibitors (HDIs) known to impair TGS, reduce *SUL* silencing in vitro at 120 parts per million (ppm) (Fig. 1C), with optimal concentrations subsequently determined at 400 and 50 ppm, respectively (Fig. 1D; Supplemental Fig. S1A,B); two additionally tested TSA analogs had similar effects (Supplemental Fig. S2). The levels of *SUL* siRNAs in SAHA- and TSA-treated *SUC-SUL* seedlings at 100 ppm were nearly identical to those of mock-treated seedlings (Fig. 1E), suggesting that HDIs inhibit *SUL* silencing at, or downstream from, the AGO effector step.

As a second lead to identifying possible modifiers of PTGS, we took into consideration that at least part of the cellular AGO1 pool exists as a peripheral membrane-associated protein (Brodersen et al. 2012), including on the endoplasmic reticulum where AGO1 mediates translational repression and some endonucleolytic cleavage (Li et al. 2013; Yu et al. 2017; Freimer et al. 2018). Accordingly, membrane-related processes, autophagy in particular, enhance AGO1 turnover and reduce its function in PTGS, in-

cluding under miRNA/siRNA deficient conditions and/or during poliovirus infections (Derrien et al. 2012). Although as yet unconfirmed, similar membrane-related processes may well underlie the lack-of-cargo-induced turnover of AGO4/AGO6 in RdDM mediated by 24-nt siRNAs (Li et al. 2006). Among the 80 "educated guess" molecules tested, Sortin1, which alters plant and yeast vacuolar markers' localization (Zouhar et al. 2004; Rosado et al. 2011), consistently enhanced *SUL* silencing in vitro at an optimal concentration of 200–400 ppm (Fig. 1C,D; Supplemental Fig. S1C). This effect was correlated with increased levels of both the 21- and 24-nt *SUL* siRNA species compared to those of mock-treated seedlings (Fig. 1E). We conclude from this preliminary analysis that the *SUC-SUL*-based experimental system exhibits sufficient consistency and robustness to enable identification of both PTGS suppressors and enhancers.

In vitro identification of robust chemical compounds that enhance the *Arabidopsis* PTGS pathway

Having established and validated our experimental settings, a medium-scale screen was initiated with an in-house (Bayer) library of patented or previously described molecules known to influence plant growth and/or adaptation to stress, two processes notoriously regulated by endogenous PTGS pathways (Khraiwesh et al. 2012; Deng et al. 2018; Singh et al. 2018). The identified *SUL* silencing modifiers were vastly dominated by enhancers representing ~8% of molecules tested, which we studied in priority; in contrast, only 1.4% of the molecules were classified as inhibitors (Fig. 2A). Two *SUL* silencing enhancers, Isoxazolone and [5-(3,4-dichlorophenyl)furan-2-yl]-piperidine-1-ylmethanethione (DFPM), stood out from this focused study due to their robust effects in vitro. At their optimal concentration range (100–350 ppm), these molecules consistently had stronger enhancing effects (55%–60% normalized chlorosis) than those of Sortin1 at its optimal concentration (35% normalized chlorosis) used as a reference PTGS-enhancer (Fig. 2B; Supplemental Figs. S1C, S3). Isoxazolone and DFPM also had highly consistent effects in independent large-scale experiments involving each ≥75 individually treated wells of four to five *SUC-SUL* seedlings (Fig. 2C,D, Supplemental Fig. S4). Further attesting to the robustness of these classes of molecules, respectively 88% and 100% of Isoxazolone and DFPM chemical analogs tested subsequently increased *SUL* silencing in seedlings' in vitro treatments, as did 56% of all Sortin1 analogs tested (Fig. 2E).

Isoxazolone, DFPM, and Sortin1 enhance RNAi in planta

To investigate if, and to which extent, the in vitro results were transposable in planta, 200 ppm of Isoxazolone,

FIGURE 2. Identification of inhibitors and enhancers of the *Arabidopsis* PTGS pathway, and in vitro validation of the robust effect of Isoxazolone and DFPM. (A) Pie chart representing the classification of all the screened molecules depending on their effect on the *SUC-SUL* reporter chlorotic phenotype. (B) Percentage of the chlorotic phenotype of *SUC-SUL* seedlings treated with the best enhancer candidates at 300 ppm selected from all the screened molecules, normalized to the mock treatment. Error bars, mean $\pm$ variation (%); $N \geq 156$. (C) Percentage of the chlorotic phenotype of *SUC-SUL* seedlings 7 d after treatment with mock or Isoxazolone, measured for ≥ 75 individual wells for two independent experiments (exp1 and exp2), normalized to one mock treatment. (D) Percentage of the chlorotic phenotype of *SUC-SUL* seedlings 7 d after treatment with mock or DFPM, measured for ≥ 81 individual wells for two independent experiments (exp1 and exp2), normalized to one mock treatment. The same sets of mock values (two independent experiments—exp1 and exp2—measured for 84 individual wells) were used for C and D. (E) Percentage of the chlorotic phenotype of *SUC-SUL* seedlings 7 d after treatment with mock, Sortin1, Isoxazolone (Isoxaz.), DFPM, or their chemical analogs (mean from 12 independent biological replicates) normalized to the mock treatment.

DFPM or Sortin1 were sprayed in parallel onto 21-d-old, soil-grown *SUC-SUL Arabidopsis* plants. The typical vein-centered chlorosis in rosette leaves was markedly increased 7 d posttreatment (dpt) with all three molecules, as compared to mock-treated plants (Fig. 3A). This effect was strictly *SUC-SUL* transgene-dependent because the *SUL* mRNA levels remained unaltered in treated nontransgenic plants (Supplemental Fig. S5A). This effect was also likely systemic because it was visible in untreated, new emerging leaves, which contained decreased *SUL* mRNA levels compared to equivalent leaves of mock-treated plants, in several independent experiments (Fig. 3A–D). Accordingly, the *SUL* protein showed increasingly reduced levels in systemic leaves of plants sprayed with Sortin1, Isoxazolone and DFPM (Fig. 3E), consistent with their observed enhanced bleaching phenotype compared to mock-treated plants.

Isoxazolone, DFPM, and Sortin1 specifically increase the DCL4 siRNA-processing activity in a probable DRB4-independent manner

The moderate, intermediate, and stronger *SUL* silencing respectively mediated by Sortin1, Isoxazolone, and DFPM, correlated with increasingly higher levels of *SUL*

siRNAs. Moreover, the DCL4-dependent 21-nt species was, accordingly, more loaded into AGO1 in Sortin1-, Isoxazolone- and DFPM-treated compared to mock-treated *SUC-SUL* rosette leaves, as assessed by immunoprecipitation (IP) with an anti-AGO1 antibody (Fig. 3F). The levels of endogenous DCL1-dependent miRNAs remained, respectively, nearly unchanged in Sortin1- and Isoxazolone-, and slightly enhanced in DFPM-treated samples, an effect also seen in AGO1 IPs (Fig. 3F). This lack of overt effects on total miRNA levels suggests that the gain in 21- and 24-nt *SUL* siRNAs is not accounted for by an increased activity of DCL1 releasing more dsRNA segments from *SUC-SUL* transcripts to be used as substrates by DCL4 and DCL3 (Henderson et al. 2006; Dunoyer et al. 2007).

To test if the molecules' effects on the artificial *SUC-SUL IR* also applied endogenously, we analyzed the 22-nt (DCL2-dependent) and 24-nt (DCL3-dependent) siRNAs derived from the previously characterized *IR71* endogenous hairpin (Henderson et al. 2006). The levels of both species were slightly enhanced by the three molecules (Fig. 3F). None of the molecules tested strongly enhanced the 24-nt siRNAs to the detriment of the 21-(DCL4, *SUC-SUL* system) or 22-nt (DCL2, *IR71*) siRNAs, which is the trademark effect of loss-of-*DRB4* function (Dunoyer et al. 2007; Brodersen et al. 2008). An effect on the recently

FIGURE 3. In planta validation and molecular analysis of the enhancer effects of Isoxazalone, DFPM, and Sortin1 on sRNA processing and loading. (A) Chlorotic phenotypes of representative *SUC-SUL* rosettes treated with mock, Isoxazalone, DFPM, or Sortin1. Scale bars: 1 cm. (B–D) qRT-PCR analyses of the *SUL* mRNA relative levels in *SUC-SUL* rosettes, treated with mock or Isoxazalone (Isoxaz., B), DFPM (C), or Sortin1 (D). Error bars, mean $\pm$ 95% confidence interval; $N = 3$. (**) $P < 0.01$, (*) $P < 0.05$ (t-test). (E) Western blot analysis of the *SUL* protein levels in *SUC-SUL* rosettes treated with mock, Isoxazalone (Isoxaz.), DFPM, or Sortin1. (Coom.) Coomassie staining used as protein loading control. Relative quantifications of *SUL* signals normalized to Coom. are indicated below the panels, with 1.00 arbitrary set for mock treatment. Similar results were obtained for two other independent experiments. (F) Western blot analysis of AGO1 total extracts and immunoprecipitates (IP) fractions of *SUC-SUL* rosettes treated with mock, Isoxazalone (Isoxaz.), DFPM, or Sortin1. (Coom.) Coomassie staining used as protein loading control. Relative quantifications of AGO1 signals are indicated below the panels, with 1.00 arbitrary set for mock treatments. These quantifications are normalized to Coom. in the total extract samples; northern blot analysis of the *SUL* siRNAs, *IR71* siRNAs, *miR160*, and *miR173* in total extracts or in AGO1-IP. (U6) Control for equal RNA loading in total extracts. For *SUL* siRNAs and *IR71* siRNAs, relative quantifications of signals are indicated below the panels, with 1.00 arbitrary set for mock treatments. These quantifications are normalized to U6 in the total extract samples. For *SUL* siRNAs, *miR160* and *miR173*, shorter or lower exposures of total extract tracks are shown to avoid saturation of signals. Similar results were obtained for one other independent experiment. (G–H) Dicing assays of *SUL* and *GFFG*-derived dsRNAs 72 h (G) or 48 h (H) after seedlings treatment with mock, Isoxazalone (Isoxaz.), DFPM, or Sortin1. (dsRNA) dsRNA not incubated with crude extract (negative control). (NT) No treatment. Relative quantifications of the 24-nt signals (in gray) or 21-nt signals (in black) are indicated below the panels, with 1.00 arbitrary set for mock treatments. Similar results were obtained for one other independent experiment.

identified DRB4-antagonists, DRB7.1 and DRB7.2, also is unlikely because both their loss-of-function and overexpression cause molecular signatures on *SUL* and *IR71* siRNAs that are distinctly different from those of the three PTGS-enhancers tested here (Dunoyer et al. 2007; Brodersen et al. 2008; Tschopp et al. 2017). Since DFPM, Isoxazolone and Sortin1 induced neither phytotoxicity nor *AtSUC2* promoter activity in treated or systemic *AtSUC2-GFP*, Col-0, or *SUC-SUL* rosette leaves (Supplemental Fig. S5B–D), we conclude that these molecules are bona fide, generic and likely DRB4-independent enhancers of *IR*-PTGS in *Arabidopsis*.

The three PTGS-enhancers had overall congruent, albeit not identical, effects on the artificial *SUC-SUL IR* and the endogenous *IR71*. The differences might be explained by the dissimilar secondary structures and DCL-processing requirements of each *IR*-derived dsRNA, the intrinsic *IR*-derived siRNA steady-state accumulation levels before treatments, and/or, possibly, contrasted *IR* expression patterns. To investigate and compare side-by-side the molecules' effects more precisely under conditions minimizing biological variations and structural differences, 2-wk-old seedlings grown in vitro were treated in liquid suspension at an effective concentration of 10 ppm (Supplemental Fig. S6). The primary effects of the molecules on de novo processed, as opposed to steady-state, siRNA levels were investigated using "dicing assays" conducted in crude extracts of the treated seedlings. *SUL*- and *GFP*-derived long dsRNAs synthesized and labeled in vitro provided independent, standardized sources of DCL substrates in this assay. Processing of both synthetic dsRNAs in extracts of untreated and mock-treated wild-type seedlings led to high and comparatively significantly lower accumulation of a DCL3-dependent 24-nt species and a DCL4-dependent 21-nt species, respectively (Fig. 3G). Strikingly, the 24-nt siRNA levels were reduced and the 21-nt siRNA levels concomitantly increased in the extracts of all PTGS enhancer-treated seedlings, which was confirmed by treatments of *dcl3* and *dcl4* loss-of-function seedlings (Fig. 3G,H). The treatments of *dcl4* mutant extracts also promoted the reduced accumulation of 24-nt siRNA from both *GFP* and *SUL* dsRNAs, ruling out that this effect in wild-type extracts results from an enhanced access of DCL4 to dsRNA relative to that of the competing DCL3 protein (Fig. 3G; Henderson et al. 2006).

Because siRNA steady-states are influenced by both their rate of processing and their loading efficacy into AGOs, the above in vitro effects could be due to increased protein levels of DCL4 and/or of AGO1, both of which are predominantly involved in the 21-nt siRNA pathway. Alternatively, or coincidentally, the levels of DCL3 and/or AGO4, predominantly involved in the 24-nt siRNA pathway, could be reduced. We thus tested the accumulation of these proteins in DFPM-, Isoxazolone- and Sortin1 -treated seedlings. We also quantified accumulation of AGO2,

known to be induced by chemical and biological stresses (Fátyol et al. 2016) and as a PTGS effector of the 21-nt siRNAs in the *SUC-SUL* system (data not shown). To simultaneously circumvent cross-hybridization problems encountered with all currently available DCL4 antibodies, we used *Arabidopsis* transgenic seedlings expressing the *DCL4:DCL4-2xFlag-2xHA* (*DCL4-FHA*) construct in the *dcl4-2* null genetic background (Supplemental Fig. S7; see Materials and Methods). Following treatments with either PTGS-enhancer, the DCL4-HA and AGO1 levels were nearly unchanged or slightly decreased (Fig. 4A,B; Supplemental Fig. S8A,B), whereas those of DCL3 and AGO4 were consistently and significantly reduced, compared to those found in mock-treated plants (Fig. 4C,D; Supplemental Fig. S8C,D). A specifically increased stability of the 21-nt siRNAs is unlikely because HEN1-mediated 2'-O-methylation—the only direct mechanism known to stabilize si/miRNAs in plants—operates on all small RNAs including 24-nt siRNAs (Li et al. 2005; Yu et al. 2005). Additionally, the levels of AGO1—which usually parallel the cellular abundance of its siRNA cargoes (Derrien et al. 2012)—were unchanged or even slightly decreased by the treatments. A specific increase in DCL4 enzymatic activity therefore likely underlies the common effects of the three PTGS-enhancers on dsRNA processing in seedling extracts and *in planta*.

The effects of DFPM on *IR*-PTGS are independent of AGO2 protein over-accumulation

DFPM, unlike Sortin1 or Isoxazolone, consistently increased AGO2 levels (Fig. 4E; Supplemental Fig. S8E) in multiple experiments. Since AGO2, in addition to AGO1, loads 21-nt *SUL* siRNAs (data not shown), we considered the possibility that parts of the effects of DFPM—the strongest of the enhancers—might relate to this specific property not seen with the other molecules. In order to test if DFPM increases AGO2 expression at the transcriptional level, we used seedlings of a transgenic line in which the AGO2 promoter (*pAGO2*) drives the expression of the *GUS* reporter gene (*pAGO2-GUS*). *pAGO2-GUS* seedlings grown in vitro were thus treated with DFPM for 2 d, or with Bleomycin, a radiomimetic compound that also induces DNA strand breaks in the *Arabidopsis* genome (Menke et al. 2001). Indeed, *Arabidopsis* AGO2 transcription was previously shown to be highly induced upon γ -irradiation, a potent inducer of DNA double-strand break (Wei et al. 2012). While, as expected, *GUS* expression was induced by bleomycin as attested by histochemical staining, neither mock- nor DFPM-treated seedlings showed enhanced *GUS* activity (Fig. 4F). These results therefore rule out that the effect of DFPM on AGO2 protein levels are mediated by enhanced AGO2 transcription and suggest, instead, an impact of the molecule at the translational and/or post-translational level. To measure the requirement for AGO2

FIGURE 4. Molecular effects of Isoxazalone, DFPM, and Sortin1 on the major silencing effectors. (A–E) Western blot analyses of the DCL4-HA (A), AGO1 (B), DCL3 (C), AGO4 (D), or AGO2 (E) protein levels in *DCL4-FHA* line #7 seedlings, 48 h after treatment with mock, Isoxazalone (Isoxaz.), DFPM, or Sortin1. (Coom.) Coomassie staining used as protein loading control. Relative quantifications of the signals normalized to Coom. are indicated below the panels, with 1.00 arbitrary set for mock treatments. Similar results were obtained for one other independent experiment (see Supplemental Fig. S8). (F) GUS staining of *pAGO2-GUS* seedlings 2 d after treatment with mock, Bleomycin (used as positive control), or DFPM. (G) Chlorotic phenotypes of the *SUC-SUL* WT and *SUC-SUL* x *ago2-1* rosettes 7 d after treatment with mock or DFPM. White lines indicate the scale bar (1 cm). (H) qRT-PCR analysis of the *SUL* mRNA relative levels in *SUC-SUL* WT and *SUC-SUL* x *ago2-1* rosettes, 7 d after treatment with mock or DFPM. Error bars, mean $\pm$ 95% confidence interval; $N = 2$. (*) $P < 0.05$ (t-test for comparison between mock and DFPM treatments in each background). (I) Western blot analysis of the *SUL* protein levels in *SUC-SUL* WT or *SUC-SUL* x *ago2-1* rosettes, treated with mock or DFPM. (Coom.) Coomassie staining used as protein loading control. Relative quantifications of the *SUL* signals normalized to Coom. are indicated below the panels, with 1.00 arbitrary set for mock treatments. Similar results were obtained for one other independent experiment. (J) Northern blot analysis of the *SUL* siRNAs, miR160 and miR173 in *SUC-SUL* WT and *SUC-SUL* x *ago2-1* rosettes, 7 d after treatment with mock or DFPM. (U6) Control for equal RNA loading. Relative quantifications of the *SUL* siRNA signals normalized to U6 are indicated below the panels, with 1.00 arbitrary set for mock treatments. Similar results were obtained for one other independent experiment.

in DFPM-mediated enhancement of *IR-PTGS*, *SUC-SUL* plants with an *ago2* null-mutant background were treated with DFPM. The increased vein-centered chlorosis and decreased *SUL* mRNA/protein levels were still observed com-

pared to mock-treated plants (Fig. 4G–I). This effect also correlated with increased *SUL* siRNA levels without any overt effect on miRNAs (Fig. 4J) as previously observed in the *SUC-SUL* plants with the wild type background

(Fig. 3F). These results suggest that the elevation in AGO2 levels has little consequence on the DFPM-dependent enhancement of *IR*-PTGS, hence further supporting DCL4 as being the primarily targeted protein by this and the two other molecules independently of DRB4 or of competitive access to dsRNA compared to DCL3.

Future research and foreseeable applications

Using *Arabidopsis* as a model system, our study provides the first proof-of-principle that chemicals can potently and specifically modulate PTGS in a whole organism. The specificity of the enhancers' action is shown by their selective effects on the 21-nt siRNA-mediators of PTGS in standardized dicing assays. Nonetheless, these assays only partially recapitulate *in planta* applications of the molecules, particularly on the *SUL* siRNAs for which accumulation of the 24-nt siRNA species was also enhanced, possibly reflecting the complex regulation of this locus including via TGS (Dunoyer et al. 2007). In single-cell organisms, genetic identification of target(s) is the logical step following the isolation of a compound displaying a desirable biological activity. We are, likewise, considering target identification of the most robust of our chemical enhancers, DFPM, via a forward-genetic screen for EMS-induced mutations that suppress its bleaching effects in *SUC-SUL* seedlings. However, we have not yet reached the stage at which we can confidently rely upon detection of such rare mutants among thousands of sprayed seedlings using the automated pipeline described here. Such a screen might be facilitated by increasing the delivery, assimilation, bio-availability and lasting-quality of these molecules with better formulations and targeted chemistry. Indeed some of the Sortin1, Isoxazolone and DFPM analogs identified in this study appear to display even better PTGS-enhancing performances than the cognate starting compounds (Fig. 2E).

Another challenge—beyond the primary scope of this proof-of-concept study—is to test if PTGS-modifiers operate in other plants. Addressing this question will likely require the development of tailored formulation/delivery methods, and might be confounded by the existence of strong genetic modifiers of PTGS in plants, found even among *Arabidopsis* ecotypes (Vaucheret 2008). Regardless, PTGS-enhancers/suppressors might facilitate *Arabidopsis* research, first as enabling tools for RNA-silencing studies, but also, more pragmatically, as modulators of phenotypes generated via experimental RNAi in this model species. Indeed, compounds can be delivered at any desired time and confined onto any desired surface by simple spray-application. Their effect is also reversible over time such that even lethal loss-of-function RNAi phenotypes could be potentially studied in single leaves or at specific developmental stages. Last but not least, the ubiquitous defensive role of DCL4-dependent vsRNAs and amenabil-

ity of *Arabidopsis* to infections by various RNA and DNA viruses now makes it possible to assess the antiviral potential of the three PTGS-enhancers or some of their derivatives. Such studies would nonetheless have to consider potential indirect effects of these compounds on plant basal, as opposed to sequence-specific, defense against pathogens given the strong ties recently uncovered for instance between endo-RNAi and *NBS-LRR* transcripts regulation (Shivaprasad et al. 2012; Boccara et al. 2014).

MATERIALS AND METHODS

Plant material

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used as wild-type (WT) control. *dcl3-1*, *dcl4-2*, and *ago2-1* *Arabidopsis* mutants have been previously described (Xie et al. 2004, 2005; Lobbes et al. 2006). *SUC-SUL* in Col-0 has been already described (Dunoyer et al. 2005), as well as *AtSUC-GFP* in Col-0 (Imlau et al. 1999). For *DCL4-FHA* transgenic lines #6 and #7, *Arabidopsis* *DCL4* promoter was amplified from Col-0 genomic DNA with pDCL4-attB4 and pDCL4-attB1r primers (Supplemental Table S1) and inserted into pDONR4-1r via Gateway BP recombination. *Arabidopsis* *DCL4* CDS (genomic) was amplified with the DCL4-attB1 and DCL4-attB2 primers (Supplemental Table S1) and inserted into pDONR221 via BP recombination. *2xFlag-2xHA* was amplified with the 2Flag2HA-attB2r and 2Flag2HA-attB3 primers (Supplemental Table S1) and inserted into pDONR2r-3 via BP recombination. *DCL4* promoter, *DCL4* CDS, and *2xFlag-2xHA* tag sequences were fused by three-way LR recombination into pB7m34GW and the final construct was transformed into Col-0 (line #6) or *dcl4-2* *Arabidopsis* mutant (line #7). Lines #6 and #7, which were selected based on their segregation ratio on Basta containing MS and homozygous T3, were used. For *pAGO2-GUS* transgenic line, *Arabidopsis* AGO2 promoter was amplified from Col-0 genomic DNA with pAGO2-attB4 and pAGO2-attB1r primers (Supplemental Table S1) and inserted into pDONR4-1r via Gateway BP recombination. *GUS* was amplified from the binary vector pMDC162 with the GUS_attB2r and GUS_attB3 primers (Supplemental Table S1) and inserted into pDONR2r-3 via BP recombination. *2xFlag-2xHA* tag was synthesized into pDONR221 (Thermo Fisher Scientific). A three-way LR recombination was performed into the binary vector pB7m34GW to fuse the AGO2 promoter, *2xFlag-2xHA* and *GUS*, and the final construct was transformed into Col-0. Based on their segregation ratio on Basta containing MS, homozygous T3 were used.

Plant growth and treatment conditions

To set up the experimental design, *Arabidopsis* seedlings were grown *in vitro* under 96-well plate format (four–five seedlings/well), on 180 μ L of MS-agar medium in growth cabinet with the following conditions: 70% humidity, 24°C, continuous light of 60 μ Einstein.m⁻².s⁻¹. Seedlings were sprayed at day 7 after germination with 5 μ L of solution containing 50–400 ppm of active molecules, 5% DMSO and EC Premix (Bayer), using an automated liquid handling Lissy platform (Zinsser). The treatment effects on *SUC-SUL* plants were recorded digitally under MacroFluo

macroscope (Leica) and quantified at day 14 by measuring the total chlorotic zone for each well with the MetaMorph software (Molecular Devices). Wells with seedlings of visually bad quality (germination or chlorotic variability) were excluded from the analyses. An average of the chlorotic zone from a minimum of four individual replicates (wells) was calculated, as well as a 95% confidence interval for each molecule. The quantification script threshold was adjusted for each plate to obtain an average of 10% of chlorotic zone for the mock treatment containing 5% DMSO and EC Premix. Subsequent chemical libraries were screened at 300 ppm with the same conditions as described above. Molecules with a putative effect on the chlorotic phenotype of the *SUC-SUL* plants, but with little or no effect on the *AtSUC2-GFP* plants (chlorosis, GFP phenotype), were next subjected to a dose-range test (from 18 ppm up to 400 ppm). An average of the chlorotic zone from a minimum of four individual replicates (wells) was calculated, as well as a 95% confidence interval for each molecule and each dose. Seedlings from 96 wells were harvested and pooled just after chlorosis quantification for molecular analyses.

For the dicing assays and western blot analyses, *Arabidopsis* seedlings were grown in vitro on MS-agar medium, pH 5.7 (MS including vitamins and MES, 5 g/L sucrose, 8 g/L Microagar - Duchefa) in growth cabinet with the following conditions: 50% humidity, 21°C, 12 h light of 30 $\mu\text{Einstein}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ /12 h dark photoperiod. Twelve-day-old seedlings were transferred into liquid MS in 6-well plates (~30 seedlings/well) for 48 h before treatment by adding the molecules directly into the medium to a final concentration of 10 ppm. Seedlings were harvested 48 h or 72 h after treatment (one well/replicate) for molecular analyses.

For experiments performed on rosettes, *Arabidopsis* plants were grown on soil in growth cabinet with the following conditions: 60% humidity, 21°C, 16 h light/8 h dark photoperiod. Molecules were resuspended in DMSO, diluted from 100 ppm to 200 ppm in water containing EC Premix, and sprayed on 4-wk-old rosettes using the EcoSpray system (Labo-Chimie France) to allow complete coverage of the leaf surface. New growth aerial tissues of four rosettes were harvested and pooled at 7 dpt for molecular analyses.

GUS staining

Briefly, treated seedlings were first fixed for 20 min in 90% acetone, and subsequently stained with 50 mM sodium phosphate (pH 7.2), 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 0.2% Triton X-100, and 2 mM X-Gluc (Roth) at 37°C in dark overnight. Staining solution was removed by ethanol washes.

RNA extraction

Total RNA was extracted from frozen, ground *Arabidopsis* tissues using Isol-RNA Lysis reagent (5-PRIME) according to the manufacturer's instructions.

Real-time qRT-PCR analyses

Total RNA was treated with DNase I (Thermo Fisher Scientific) for 50 min at 37°C before reverse transcription with the RevertAid

First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Real-time qPCR was performed on a LightCycler480 II (Roche) with the KAPA SYBR FAST qPCR Kit (KAPA Biosystems) and gene-specific primers (Supplemental Table S2). PCR was done in 384-well optical reaction plates heated at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 20 sec, and elongation at 72°C for 40 sec. A melting curve was performed at the end of the amplification by steps of 1°C (from 50°C to 95°C). Threshold cycle (C_t) values were determined by calculating the second derivative maximum of three technical replicates per sample. Relative expression levels were calculated by subtracting the average C_t values for the genes of interest to those of *At4g26410* (Czechowski et al. 2005) (to give ΔC_t) and then calculating " $2^{\Delta C_t}$ ". Normal distributions were confirmed by Shapiro–Wilk test ($\alpha = 0.02$). Equal variances were tested by *F*-test (95% confidence interval). *P*-values were calculated using *t*-test (one-tailed distribution and two-sample unequal variance if the equal variance was not validated; one-tailed distribution and two-sample equal variance if the equal variance was validated).

RNA blot analyses

Northern analyses of low molecular weight RNA were performed with 10–50 μg of total RNA diluted in 50% formamide. Total RNA was separated in 0.5× TBE on a 17.5% polyacrylamide-urea gel, electrotransferred in 0.5× TBE to a HyBond-NX membrane (GE Healthcare Life Sciences) and crosslinked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma-Aldrich)—mediated chemical crosslinking for 90 min, as previously described (Pall and Hamilton 2008). RNA blots were prehybridized in PerfectHyb Plus Hybridization Buffer (Sigma-Aldrich) at 42°C for 30 min before adding probes of interest. Random-radiolabeled probes (Supplemental Table S3) were generated by incubating PCR fragments with the Prime-a-Gene labeling system (Promega) in the presence of [α - ^{32}P]-dCTP (Hartmann Analytic). Oligonucleotide probes (Supplemental Table S4) were end labeled by incubation with T4 PNK (Thermo Fisher Scientific) in the presence of [γ - ^{32}P]-dATP (Hartmann Analytic). Membranes were probed overnight at 42°C and then washed three times with 2× SSC, 2% SDS at 50°C. Radiolabeled signals were detected either by autoradiography (x-ray films) or by phosphor-imaging scanning on a Typhoon FLA900 (GE Healthcare Life Science). Membranes were probed several times with multiple sequences after stripping with boiling 0.1% SDS. U6 was probed together with miR173 in Figure 3F or miR159 in Figure 4J, though different exposure times (Fig. 3F) or levels (Fig. 4J) were used to prepare the figures. Signal intensities were quantified using ImageJ software (<http://imagej.nih.gov/ij>).

Total protein extractions and western blot analyses

For SUL and GFP protein analyses, total proteins were precipitated overnight from the phenolic phase of the Isol-RNA Lysis in 75% ice-cold acetone, washed twice with 80% ice-cold acetone and resuspended in 3% SDS, 62.5 mM Tris-HCl, pH6.8, 10% glycerol (v/v). For HA, AGO1, AGO2, AGO4, and DCL3 protein analysis, total proteins were extracted by following the Tanaka method (Hurkman and Tanaka 1986).

Protein concentrations were determined by a modified Lowry procedure using the DC Protein Assay Kit (Bio-Rad). Proteins were resolved by SDS-PAGE and electro-transferred to Immobilon-P PVDF membrane (Millipore). Following 1 h blocking step in 1× TBS + 0.1% Tween-20 supplemented with 5% nonfat dry milk, antibody incubations were carried out overnight at 4°C with constant shaking. Primary antibodies were used following dilutions in Supplemental Table S5. Membranes were next washed three times in TBS + 0.1% Tween-20, and subsequently incubated for 1 h with HRP-conjugated goat anti-rabbit antibodies (except for HA detection), then rinsed three times before detection with ECL Western Blotting Detection Kit (GE Healthcare Life Science). Membranes were finally stained with Coomassie blue for equal loading control. Signal intensities were quantified using ImageJ software (<http://imagej.nih.gov/ij>).

Immunoprecipitations (IPs)

Arabidopsis ground tissues were mixed 1/3 (g/mL) with IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% NP-40) containing 2 µM proteasome inhibitor MG-132 and cOmplete protease inhibitor cocktail (Roche), and incubated for 30 min at 4°C on a rotating wheel. All the following steps were performed at 4°C or on ice. Cell debris were removed by centrifugation for 30 min at 8000g. Supernatant was precleared by incubating for 1 h with 30 µL of Protein A-agarose beads (Sigma). Beads were removed by centrifugation for 1 min at 1500g, and supernatant was incubated with anti-AGO1 antibody at 1/500 dilution for 1 h. 30 µL Protein A-agarose beads were added for 1 h and 30 min. Beads were pelleted, washed three times in IP buffer, and resuspended in 1 mL of Isol-RNA Lysis reagent. Both RNA and proteins were extracted, as previously described.

Dicing assays

SUL-derived sequences were prepared from Col-0 gDNA. *GFFG*-derived sequences were amplified from a eGFP containing vector (pK7WGF2). Sense and antisense sequences were amplified using primers containing the T7 promoter sequence (Supplemental Table S6) and in vitro transcribed into RNA by T7 RNA polymerase (Thermo Fisher Scientific) in the presence of [α -³²P]-CTP (Hartmann Analytic). Remaining DNA was removed by DNase treatment (Thermo Fisher Scientific). Transcripts were cleaned first on a mini Quick Spin Columns (Roche) and subsequently by phenol-chloroform extraction. Equal amounts of sense and antisense ³²P-labeled RNAs were annealed in annealing buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl) by placing the tubes in a heating block preheated at 95°C until cooling down to room-temperature. The quality of the dsRNAs was verified by loading 1 µL onto a 5% native acrylamide gel.

In vitro dicing assays were performed, as previously described (Qi et al. 2005). *Arabidopsis* seedlings were ground into fine powder with liquid nitrogen and homogenized into extraction buffer (1 g/mL—20 mM Tris-HCl, 4 mM MgCl₂, pH 7.5) containing 5 mM DTT and protease inhibitor cocktail (Roche). Cell debris were removed by centrifugation at 22,000g at 4°C for 20 min. The supernatant was collected and total protein concentration was determined using the DC Protein Assay Kit (Bio-Rad) before being diluted to 0.5 mg/mL.

³²P-labeled dsRNAs (around 50,000 counts per min) were incubated for 2 h at 25°C in a 20 µL reaction containing 15 µL of crude extract (10–20 µg of total protein) and 4 µL of 5× Dicer reaction buffer (0.5 M NaCl, 5 mM ATP, 1 mM GTP, 6 mM MgCl₂, 125 mM creatine phosphate, 150 µg/mL creatine kinase and 2 units RNase Inhibitor). The reaction was stopped by adding 250 µL of Isol-RNA Lysis reagent and RNA was extracted according to the manufacturer's instructions, with addition of glycogen during the RNA precipitation step. RNA was resuspended in formamide 50%, resolved on a 15% denaturing PAGE gel, electrotransferred to a HyBond-NX membrane (GE Healthcare Life Science) and crosslinked by UV irradiation. Radiolabelled signals were detected by phosphor-imaging scanning on a Typhoon FLA900 (GE Healthcare Life Science).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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