The Plant Journal (1999) **17**(5), 461–466

The following paper was submitted to *The Plant Journal.* After peer review and revision it was accepted. Following the discovery of scientific fraud in the Department of Genetic Principles of Plant Breeding at the Max Planck Institute for Plant Breeding in Köln, a wide-ranging group of researchers was assembled to repeat some key experiments. The outcome, described in the paper below, is that the published data from the Köln MPI on phytohormone-independent cell division were not reproducible. This concerns papers dating back to 1992.

Re-evaluation of phytohormone-independent division of tobacco protoplast-derived cells

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Summary

We have used a [^{3}H] thymidine incorporation assay and microscopic observation in order to reassess recently published data dealing with the response of tobacco protoplasts to phytohormones, lipochitooligosaccharides and peptides (Harling *et al.*, 1997; Hayashi *et al.*, 1992; Miklashevichs *et al.*, 1996; Miklashevichs *et al.*, 1997; Röhrig *et al.*, 1995; Röhrig *et al.*, 1996; van de Sande *et al.*, 1996; Walden *et al.*, 1994). These proliferation assays reveal that, in contrast to published data, isolated cells of the investigated mutant plant lines axi159 (Hayashi *et al.*, 1992; Walden *et al.*, 1994), axi4/1 (Harling *et al.*, 1997) and cyi1 (Miklashevichs *et al.*, 1997), which were generated by activation T-DNA tagging, were unable to grow in the absence of auxin or cytokinin. Furthermore, lipochitooligosaccharides which play a key role in the induction of nodules on roots of legumes were unable to promote auxin- or cytokinin-independent cell division in tobacco protoplasts as claimed by Röhrig *et al.* (1995, 1996). The finding of van de Sande *et al.* (1996) that *ENOD40* confers tolerance of high auxin concentration to wild-type tobacco protoplasts was also reinvestigated. The results of our investigations show that we were unable to reproduce the proliferation data presented in this study, which were obtained by counting tobacco protoplast-derived cells undergoing division. In total, none of the published data on phytohormone-independent division of tobacco cells could be reproduced.

Received 27 October 1998; revised 23 December 1998; accepted 7 January 1999.
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Introduction

This paper reports on the use of a new assay to re-examine earlier published work dealing with the hormonal control of tobacco cell division (Harling *et al.*, 1997; Hayashi *et al.*, 1992; Miklashevichs *et al.*, 1996; Miklashevichs *et al.*, 1997; Röhrig *et al.*, 1995; Röhrig *et al.*, 1996; van de Sande *et al.*, 1996; Walden *et al.*, 1994). In these papers, the division frequency of isolated tobacco cells was preferentially determined by microscopic counting of cells undergoing proliferation. In order to independently test these data, we used cell division assays which are based either on the incorporation of [^{3}H] thymidine into the DNA of proliferating protoplast-derived cells or on the ability of dividing cells to form microcalli after embedding in agarose. Here we report on the reproducibility of data in the published work in question.

Results and discussion

Cultured tobacco protoplasts have been used to dissect the response of plant cells to phytohormones, peptides and lipochitooligosaccharides (LCOs) as a novel class of plant growth regulators. Under defined culture conditions, these cells proliferate and form calli when the phytohormones auxin and cytokinin are added to the medium. The aim of these publications brought into question was to identify growth factors or genes that promote phytohormone-independent cell division. To assess the effect of the expression of such genes on the phytohormone response, the proliferation of wild-type protoplast-derived cells in

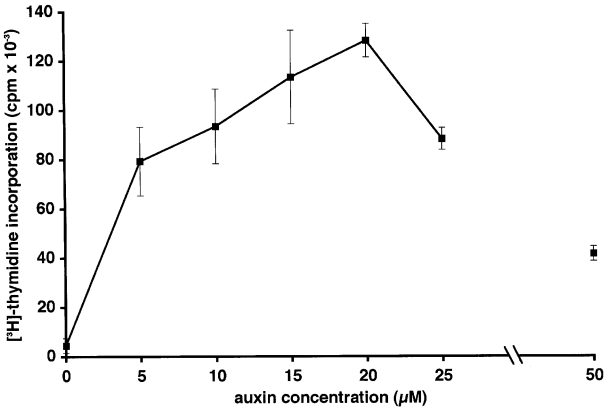

Figure 1. Effect of different auxin concentrations on the proliferation of tobacco mesophyll cells.
Protoplast-derived cells (5×10^5) were incubated in the presence of 1 μM kinetin and varying amounts of 1-NAA for 5 days. After pulse labelling with [^{3}H]-thymidine for 24 h, the [^{3}H]-thymidine incorporation into DNA of isolated tobacco cells was assayed as described in Experimental procedures. This experiment was repeated twice with similar results.

the absence of externally supplied phytohormones was compared to that of transfected protoplasts or protoplasts made from transgenic plants. Since there is a close correlation between cell doubling and DNA synthesis, we used the incorporation of tritiated thymidine into cellular DNA as an indirect parameter of cell proliferation. This [^{3}H] thymidine incorporation assay was developed and used in addition to the assay which had been used throughout the previously published work, and which was based on the counting of dividing cells.

Effect of different auxin concentrations on cell division

Protoplasts were incubated in the presence of 1 μM kinetin and different concentrations of auxin ranging from 5 to 50 μM of 1-NAA. After 5 days, the cells were pulse-labelled with [^{3}H] thymidine for 24 h, and the incorporation of radioactivity into trichloroacetic acid (TCA) precipitable material was determined by scintillation counting. As expected, the addition of auxin and cytokinin to cultured wild-type protoplasts synergistically activated DNA synthesis in these cells (Figure 1). Cytokinin alone was not sufficient to stimulate the incorporation of radioactivity into cellular DNA. The results obtained by the [^{3}H] thymidine incorporation assay correlated well with data obtained by microscopic visualisation of tobacco cells and were essentially similar to those obtained by evaluating the number of calli which appeared after the embedding of dividing protoplast-derived cells in agarose (data not shown). The auxin dose–response curve (Figure 1) showed that division in wild-type tobacco cells is stimulated by a relatively broad range of auxin concentrations (5–25 μM of 1-NAA). A significant sensitivity to higher levels of auxin in the medium was only observed in the presence of 50 μM 1-NAA. These findings are in contrast to data presented in previous publications (Ichikawa *et al.*, 1997; Miklashevichs *et al.*, 1997; van de Sande *et al.*, 1996; Walden *et al.*, 1994), which reported that wild-type (SR1) tobacco cells require a defined amount of 5 μM 1-NAA for optimal cell division. Furthermore, the considerably diminished division frequency of tobacco cells in the presence of 13.8 μM of 1-NAA shown in these papers is not in line with the present findings.

Proliferation of 'mutant tobacco cells' generated by activation T-DNA tagging

In several publications, activation T-DNA tagging was used to generate three different mutant cell lines from which plants were regenerated by selecting for growth in the presence of hygromycin and in the absence of externally supplied phytohormones (Harling *et al.*, 1997; Hayashi *et al.*, 1992; Miklashevichs *et al.*, 1997; Walden *et al.*, 1994). This method is based on the use of a T-DNA tag which contains multiple transcriptional enhancers (Walden *et al.*, 1991). The integration of the tag into the plant genome should produce a dominant mutation which leads to a deregulated expression of flanking plant genes. Using activation T-DNA tagging, cytokinin-independent mutants of *Arabidopsis* have indeed been generated which allow the identification of a gene involved in cytokinin signal transduction (Kakimoto, 1996), thus confirming the general validity of the activation gene tagging approach. However, it was reported that cultured cells isolated from the tagged mutant plants cyi1 (Miklashevichs *et al.*, 1997), axi4/1 (Harling *et al.*, 1997) and axi159 (Hayashi *et al.*, 1992), in which the gene *AXI1* was tagged (Walden *et al.*, 1994), were able to divide in the absence of externally supplied auxin. In addition, protoplast-derived cells from cyi1 plants were claimed to divide without exogenously added cytokinin and auxin (Miklashevichs *et al.*, 1997). To re-evaluate these results, wild-type tobacco SR1 and mutant plant lines cyi1, axi4/1 and axi159 were grown as described under Experimental procedures. From 8-week-old plants, protoplasts were isolated and analysed for their phytohormone requirement. The data from the [^{3}H] thymidine incorporation assays clearly show that tobacco cells from cyi1 mutant plants were unable to proliferate in the absence of auxin or cytokinin. Interestingly, these cells did not divide even in the presence of both phytohormones during the assay period described (Miklashevichs *et al.*, 1997). Furthermore, cells isolated from transgenic axi4/1 and axi159 tobacco plants did not incorporate [^{3}H] thymidine in the absence of auxin (Figure 2). In addition, growth and division was scored by microscopic evaluation of the cells 3, 5, 7 and 9 days after preparation of protoplasts and by the ability of dividing plant cells to form microcalli. The results from these analyses confirmed that the investigated cell lines

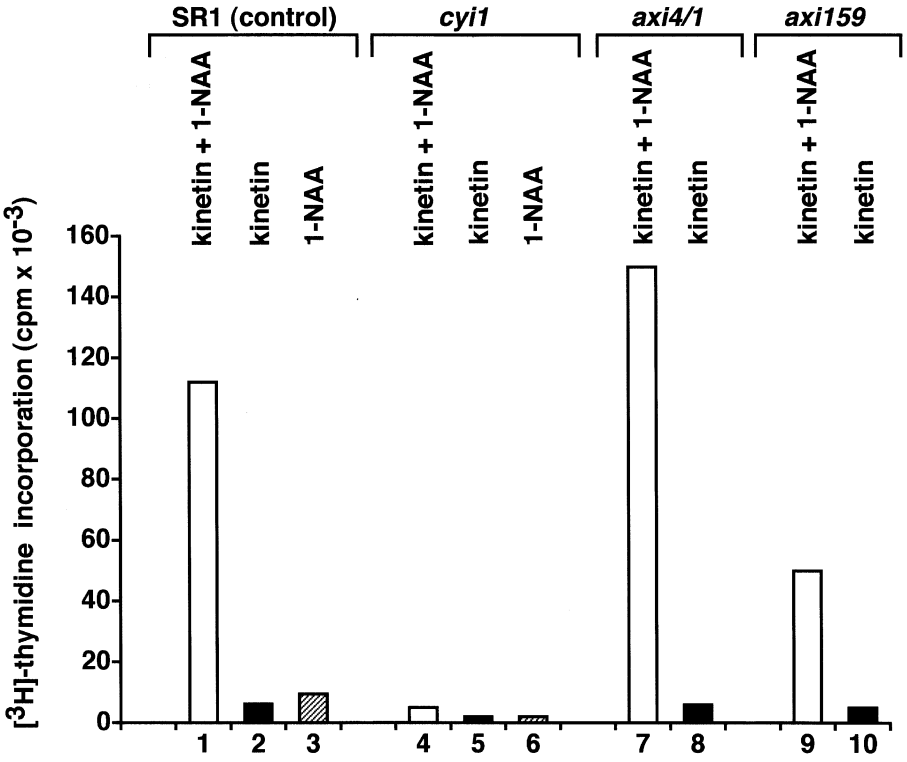

Figure 2. Proliferation of mutant cells generated from the tobacco cell lines *cyi1*, *axi4/1* and *axi159*.
Cells were grown in the presence of growth factors as indicated at the top of the figure. Concentrations of phytohormones in the media were: 1 μM kinetin plus 5 μM 1-NAA (open bars), 1 μM kinetin alone (solid bars), 5 μM 1-NAA alone (hatched bars). Conditions and measurements of [^{3}H]-thymidine incorporation were as in Figure 1. This experiment was repeated twice with similar results. Standard deviations were, on average, 13% of the means shown here.

were unable to divide in the absence of externally supplied auxin or cytokinin (data not shown).

Functional analyses of DNA which was tagged in 'mutant plants'

In publications by Hayashi *et al.* (1992), Harling *et al.* (1997) and Miklashevichs *et al.* (1997) it was reported that overexpression of plant DNA sequences flanking the T-DNA tag in the plant genome is responsible for the so-called 'phytohormone-independent' growth of the mutant tobacco cells. To demonstrate this, plant DNA carrying the T-DNA tag, together with an origin of replication and an antibiotic marker gene functional in *Escherichia coli*, was rescued as described by Walden *et al.* (1991). It was claimed that this rescued plant DNA was able to confer phytohormone-independent cell division to wild-type tobacco protoplast-derived cells after polyethylene glycol (PEG)-mediated transfection. To re-analyse the biological activity of the rescued sequences, PEG-mediated DNA uptake experiments were performed following the protocols published in the original papers. The rescued plasmids, pHH159 derived from 'mutant' plant axi159, p19En4 from axi4/1, and pCYI1 from cyi1 were transfected into SR1 protoplasts. These transfected cells were then tested for their ability to divide in the absence of auxin or cytokinin. Our results show that, in contrast to earlier claims, cells transfected with rescued DNA were unable to divide and form microcalli in the absence of externally supplied phytohormones. Furthermore, Hayashi *et al.* (1992), Harling *et al.* (1997) and Miklashevichs *et al.* (1997) used the rescued plant DNA as hybridization probes to isolate full length cDNAs corresponding to the regions in question which were subsequently subcloned into expression vectors. We re-introduced these constructs into wild-type protoplasts by transfection and found that the cDNA constructs could not confer phytohormone-independent growth to tobacco cells (data not shown).

Effect of LCOs on growth of tobacco cells in the absence of auxin or cytokinin

In the paper by Röhrig *et al.* (1995), it was reported that synthetic lipochitooligosaccharides (in a concentration range of 10^{-7} to 10^{-15} M) alleviate the requirement for auxin and cytokinin to sustain the growth of tobacco cells. The LCOs synthesized *in vitro* in these experiments were biologically active, as confirmed by their ability to deform root hairs of vetch which is a specific bioassay for nodulation factors (Heidstra *et al.*, 1994).

To test the response of non-leguminous plant cells to LCOs, we isolated protoplasts from wild-type tobacco and cultured these cells in the presence of LCOs but in the absence of kinetin or 1-NAA. The results presented in Figure 3 show that LCOs added at a concentration of 10^{-7} M to cultured tobacco cells could replace neither auxin nor cytokinin. Furthermore, Northern blot data presented in the paper by Röhrig *et al.* (1995), showing that LCOs and

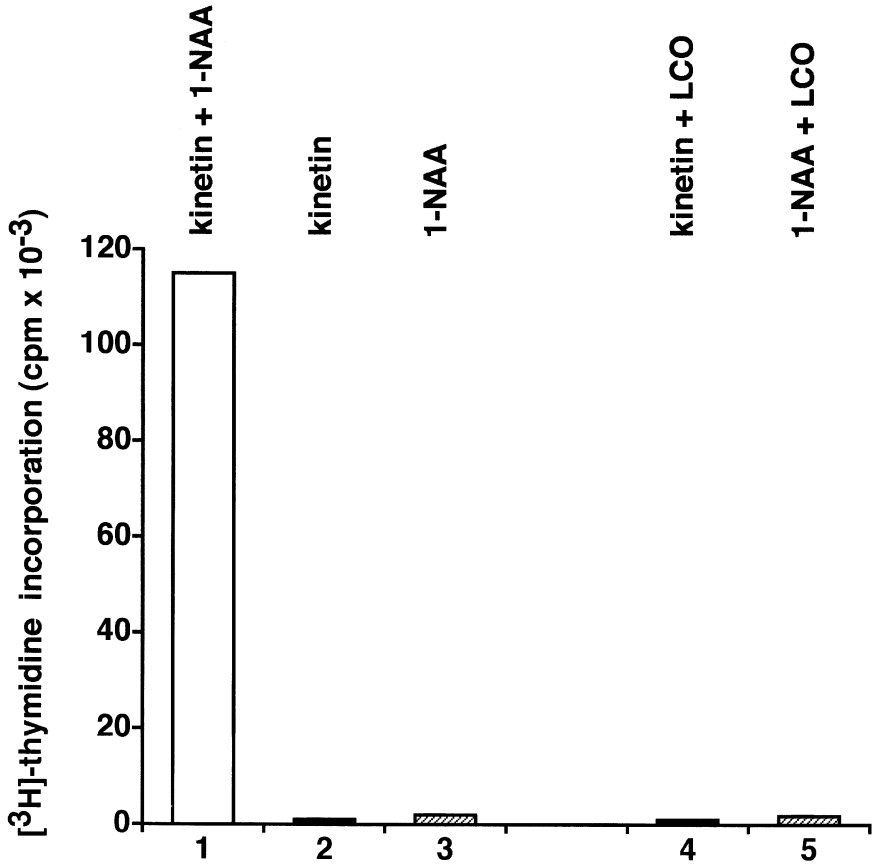

Figure 3. Effect of synthetic LCOs on the proliferation of isolated SR1 cells. The response of cultured tobacco cells to 10^{-7} M of N-*trans*-9-octadecenoyl GlcN (β-1–4-GlcNAc)$_3$ in the presence of 1 μM kinetin (solid bars) or 5 μM 1-NAA (hatched bars) was determined using the [^{3}H]-thymidine incorporation assay. As control, isolated tobacco cells were grown in a medium containing both phytohormones (open bar). This experiment was repeated twice with similar results.

auxin activate the expression of the *AXI1* gene in isolated tobacco cells, could not be reproduced (data not shown).

To test the putative effects of different growth factors on *AXI1* expression, we also used the chimeric P_{AXI}-*GUS* expression plasmid in which the reporter gene *GUSA* was fused to the *AXI1a* promoter from *Arabidopsis thaliana* (Röhrig *et al.*, 1996). Tobacco protoplasts transfected with the reporter gene plasmid were incubated in the presence of kinetin at 10^{-6} M and additionally with LCOs at 10^{-7} M or 1-NAA at 5×10^{-6} M. After 48 h, neither LCOs nor 1-NAA was found to stimulate transient *GUS* expression (data not shown), which is in agreement with the above data showing that neither LCOs nor auxin are able to induce the *AXI1* promoter.

Do plant cells release a peptide as a mediator of the auxin signal?

Recent work by Miklashevichs *et al.* (1996) has suggested that auxin-induced division of plant cells might be mediated by a peptide. In this paper, the putative anti-auxin, β-naphthalene acetic acid (2-NAA; Macdonald *et al.*, 1991; Watahiki *et al.*, 1995) was used to inhibit auxin-triggered cell division. Using the [^{3}H] thymidine proliferation assay, we analysed the effect of different 2-NAA concentrations on the division of isolated tobacco cells in the presence of kinetin. The results showed that 2-NAA stimulates cell growth as well as the auxin 1-NAA and thus cannot be used to inhibit auxin-induced proliferation of tobacco cells (data not shown). The experiments published by Miklashevichs *et al.* (1996) showed that, in response to the 1-NAA treatment, tobacco cells might release a second growth-promoting factor (presumably a peptide) that mediates the growth-promoting effects of auxin. Since 2-NAA was the basic tool to inhibit cell division in these investigations, the data presented by Miklashevichs *et al.* (1996) are questionable.

Does the ENOD40 *peptide modify the auxin response of tobacco cells?*

It has been demonstrated that *ENOD40* encodes a small peptide and that overexpression of *ENOD40* in tobacco plants leads to the formation of additional side shoots (van de Sande *et al.*, 1996). Furthermore, it was reported that *ENOD40* peptides from legumes and tobacco confer tolerance of high auxin concentrations to tobacco protoplast-derived cells. In this study, the inhibitory level of auxin in the assay was defined to be 13.8 μM of 1-NAA. However, tobacco cells exhibit optimal rates of cell proliferation

between 5 and 25 μM of auxin as measured by [^{3}H] thymidine incorporation (Figure 1) and, therefore, the response of tobacco cells to *ENOD40* was re-investigated. The auxin response of protoplasts generated from wild-type tobacco and transgenic plants expressing soybean *ENOD40* was compared. Second, the effect of the addition of synthetic tobacco and soybean peptides, as well as the transient expression of *ENOD40* on the proliferation of tobacco cells was measured. Using the [^{3}H]-thymidine-based cell division assay, we were unable to reproduce the proliferation data which had been obtained by counting tobacco cells undergoing division (data not shown).

Conclusion

Our data on [^{3}H] thymidine incorporation show that auxin and cytokinin are required for the cell division of so-called axi and cyi mutants and, although one can tag genes by 'gain of function' T-DNA tagging, this did not lead to the isolation of phytohormone-independent mutants. Although small peptides encoded by the *ENOD40* gene may play a very important role in plant development, our data do not support the notion that these small peptides render tobacco cells insensitive to high concentrations of auxin.

Experimental procedures

Plant material and DNA

Nicotiana tabacum Petit Havanna SR1 (Maliga *et al.*, 1975) was used as the wild-type plant. Mutant plants representing a homozygous F3 generation of axi159 (Hayashi *et al.*, 1992), axi4/1 (Harling *et al.*, 1997) and cyi1 (Miklashevichs *et al.*, 1997) were grown in the presence of hygromycin (15 mg l^{-1}) and correspond to those used in the published work. The homozygous F4 generation of mutant 11S plants expressing 35S-*GmENOD40–2* (van de Sande *et al.*, 1996) were selected on methotrexate (0.1 mg l^{-1}).

Plant DNA was prepared as described previously (Edwards *et al.*, 1991) and analysed for the presence of T-DNA by PCR using the following primer combinations:

(i) 5′ – GAT ATC TAG ATC CGA AAC TAT CAG – 3′ and
5′ – GTG ATA GAT CAT ACG TAG GTC GAT – 3′ for axi159
(ii) 5′ – CTT CAA TCG TTG CGG TTC TGT CAG – 3′ and
5′ – GCA TTC AGT GCT GCA CAG CAG AG – 3′ for axi4/1
(iii) 5′ – GAT ATC TAG ATC CGA AAC TAT CAG – 3′ and
5′ – GAT ATC TAG ATC CGA AAC TAT CAG – 3′ for cyi1.

Specific amplification of sequences containing T-DNA and flanking plant sequences resulted in unique fragments of 0.9 kb for the mutant axi159, 2.2 kb for axi4/1, and 1.2 kb for cyi1.

DNA of rescued plasmids derived from axi and cyi mutants was analysed by restriction digestion and compared with data presented in the original publications.

Cell culture

Mesophyll-protoplasts isolated from *Nicotiana tabacum* SR1 leaf tissue (Maliga *et al.*, 1973) were washed twice in K3 medium (Nagy and Maliga, 1976) containing 0.4 M sucrose and resuspended in the same medium to a final density of 10^5 cells ml^{-1}. Growth-promoting compounds like α-naphthalene acetic acid (1-NAA, Sigma), β-naphthalene acetic acid (2-NAA, Sigma), cytokinin (kinetin, Sigma) and synthetic LCO (*N-trans*-9-octadecenoyl GlcN [β-1,4 GlcNAc)$_3$ were added as indicated. Tobacco protoplasts were cultured in the presence of growth factors for 2 days in the dark at 26°C, followed by 3 days in continuous dim light (26°C).

Cell proliferation assays

Fifty μl of a diluted [^{3}H] thymidine solution (5 μCi) was added to isolated tobacco cells cultured for 5 days, and cells were re-incubated for 24 h. The diluted [^{3}H] thymidine solution (100 μCi ml^{-1}) was prepared from a 1 mCi ml^{-1} stock (Biotrend) by dilution 1:10 with a sterile unlabelled thymidine solution (10^{-5} M). After radiolabelling of the cells, the suspension was diluted 1:1 with the washing solution W5 (Menczel *et al.*, 1981) and cells were harvested by centrifugation (Hettich centrifuge, 1000 g for 7 min). The supernatant was removed and cells were resuspended in 1 ml of ice-cold 5% TCA. The tubes were kept on ice for 30 min. To remove acid-soluble radioactivity, cells were centrifuged and washed separately with cold 5% TCA and absolute ethanol. Wet cell pellets were then solubilized in 500 μl of 0.1 M NaOH containing 0.2% SDS for 30 min at 37°C. Radioactivity incorporated into TCA-insoluble material was determined by scintillation counting using an acidified scintillation cocktail.

In addition to the assay based on [^{3}H] thymidine incorporation into the DNA of proliferating cells, division of isolated tobacco cells was evaluated microscopically 5 days after the addition of growth factors to the medium.

Alternatively, the ability of dividing cells to form microcalli within agarose sectors floating in liquid medium (Negrutiu *et al.*, 1987) was used as a further method to measure cell proliferation.

Transient expression assays

Protoplasts were transfected with 10 μg of different DNA-constructs using PEG4000 treatment (Negrutiu *et al.*, 1987). To monitor the activation of the *AXI1* promoter, cells were transfected with the P_{axi}-*GUS* plasmid and after 48 h transiently expressed GUS was determined as described by Jefferson *et al.* (1987).

Acknowledgements

We thank Elizabeth Schell for comments and critical reading of the manuscript.

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