Agrobacterium-mediated inoculation of asymptomatic Apple latent spherical virus as gene silencing vector in pea (Pisum sativum L.)

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Abstract
Apple latent spherical virus (ALSV) has been developed into a virus-induced gene silencing vector with a broad host range, including legumes. Using Agrobacterium tumefaciens, delivered via stem injection, we introduced ALSV vectors carrying a phytoene desaturase (PDS) sequence from Phaseolus vulgaris, resulting in highly uniform knockdown phenotypes on Pisum sativum L., 23 days postinoculation. The infection rate ranged from 7.6% to 36.3% on five different P. sativum varieties using Agrobacterium stem inoculation. Mechanical inoculation using infected pea sap improved the infection rates to 80% on P. sativum cultivar AAC Lacombe. Reverse transcription polymerase chain reaction and real-time reverse transcription polymerase chain reaction demonstrated that ALSV virus RNA accumulated in roots, tendrils and leaves, accompanied by decreased PsPDS (Pisum sativum phytoene desaturase) expression level. ALSV virus RNA was also detected from sepals, petals, carpels, pods, and seed coats but not in embryos. This method simplifies the ALSV inoculation and improves the use of ALSV vectors for functional genomics in P. sativum.

KEYWORDS
Apple latent spherical virus, Agrobacterium-mediated inoculation, Pisum sativum, virus-induced gene silencing

1 | INTRODUCTION

Virus-induced gene silencing (VIGS) has been utilized to analyze and characterize the functions of known and unknown gene products in plants (Baulcombe, 2004; Liu, Schiff, & Dinesh-Kumar, 2002; Lu, Martin-Hernandez, Peart, Malcuit, & Baulcombe, 2003). However, VIGS has not been broadly employed in legume plants because of the limitations associated with virus inoculation efficiency. In the interest of sustainable agriculture, legumes are increasingly being recognized as desirable crops because of their contribution to human dietary protein and their ability to form nitrogen-fixing symbioses with members of the Rhizobiaceae family. With increasing availability of genomic data for a number of legumes, it is necessary to develop effective approaches to facilitate investigation of gene function to improve plant yield, resistance to pests and diseases, abiotic stress tolerance, and food quality. VIGS could be a simple and efficient approach for high-throughput functional genomics in legume plants because it has several advantages compared to other approaches: VIGS can decrease the expression of one or more genes without time- and labor-intensive tissue culture or generating
transgenic plants; VIGS has the potential to generate observations associated with novel phenotypes in a single generation; VIGS can silence a series of genes with functional redundancy by including a highly conserved region of a gene family as a target sequence in the viral vector; and finally, VIGS allows targeting of genes that may not produce plants when knocked out or mutated, such as those that result in embryo lethality (Benedito, Visser, Angenent, & Krens, 2004; Burch-Smith, Anderson, Martin, & Dinesh-Kumar, 2004; Godge, Pursayastha, Dasgupta, & Kumar, 2008; Hettenhausen, Baldwin, & Wu, 2014; Robertson, 2004).

Several VIGS vectors have been used for gene silencing in legume plants. Bean pod mottle virus (BPMV) has been used to silence specific genes in soybean (Glycine max L. Merr.), common bean (Phaseolus vulgaris L.) and pea (Pisum sativum L.) successfully (Meziadi et al., 2016; Zhang, Bradshaw, Whitham, & Hill, 2010; Zhang & Ghabrial, 2006; Zhang, Yang, Whitham, & Hill, 2009). BPMV has been variously delivered by particle bombardment, direct DNA leaf rubbing, or mechanical rubbing with infected leaf sap. BPMV has proven a valuable tool for gene silencing as well as for expression of gene sequences (Zhang, Whitham, & Hill, 2013). Pea early browning virus (PEBV) infectious clones have been used for gene silencing in *P. sativum*, introduced by Agrobacterium-mediated infiltration (Constantin et al., 2004). Cucumber mosaic virus (CMV)-based vectors silenced genes involving in flavonoid biosynthesis in soybean (Nagamura et al., 2007). Lim et al. (2016) developed a VIGS vector based on the soybean yellow common mosaic virus for use in soybean.

Apple Latent Spherical Virus (ALSV) based vectors hold considerable promise for legume functional studies. ALSV is composed of two single-stranded RNA genomes (RNA1 and RNA2; Li et al., 2000). A number of ALSV infectious clones have been generated, pEALSR1/pEALSR2 in *pE18PGT* with an enhanced CaMV 35S promoter, pCALSR1/pCALSR2-XSB in *pCAMBIA1300* vector, and pBICAL1/pBICAL2 in *pBCIP35* vector under the control of the CaMV 35S promoter (Kawai et al., 2014; Kon & Yoshikawa, 2014; Li, Sasaki, Isogai, & Yoshikawa, 2004). Compared with BPMV, pea early browning virus, and CMV gene silencing vectors, ALSV has several advantages. First, the ALSV VIGS vector does not cause obvious symptoms of infection in most plants tested to date, which may otherwise confound interpretation of any phenotype that arises from silencing the target (Igarashi et al., 2009; Li et al., 2004). Because of this, ALSV may be used as a vaccine to prevent subsequent infection from other viruses such as BPMV. *Zucchini mosaic virus*, CMV, and Tospoviruses (Satoh et al., 2014; Taki, Yamagishi, & Yoshikawa, 2013; Tamura et al., 2013). Second, ALSV VIGS vectors have demonstrated a broad range of potential hosts, including *Nicotiana* species (*Nicotiana tabacum*, *Nicotiana occidentalis*, *Nicotiana glutinosa*, and *Nicotiana benthamiana*), *Solanum lycopersicum*, *Arabidopsis thaliana*, *Cucurbitaceae*, legume species, and the Rosaceae family (Igarashi et al., 2009; Kawai et al., 2014).

In addition, no insect vector of ALSV transmission has been identified, minimizing the risk of cross-contamination between plants. ALSV has been introduced to legume plants by rub-inoculating after two rounds of *Chenopodium quinoa* infection (Igarashi et al., 2009). Another procedure involves extracting RNA from *N. benthamiana* leaves for microparticle bombardment of soybean leaves, which requires specialized equipment (Yamagishi & Yoshikawa, 2009). Concentrated ALSV-infected *N. benthamiana* sap was used by Satoh et al. (2014) to inoculate pea as a vaccine, however this means an additional step instead of direct inoculation of the target species. Recently, agro-infiltration of *N. benthamiana* leaves was used prior to inoculating soybean (Gedling et al., 2018).

Inoculation with Agrobacterium tumefaciens constitutes a common method to introduce viruses into plants, allowing it to be broadly and quickly used to identify putative gene functions by VIGS (Vaghchhipawala, Rojas, Senthil-Kumar, & Mysore, 2011). Different agro-inoculation approaches have been developed such as using a toothpick with an Agrobacterium colony to directly infect a seedling leaf (Lu et al., 2003), syringe infiltration (Liu et al., 2002), and vacuum infiltration (Ekengren, Liu, Schiff, Dinesh-Kumar, & Martin, 2003). Nevertheless, agro-mediated inoculation to deliver viruses to legume plants has not been applied broadly and remains limited by its relative inefficiency.

Here, we tested several inoculation approaches and developed a simple inoculation method using a syringe needle by injecting Agrobacterium carrying ALSV VIGS vectors into the stem of *P. sativum* seedling. ALSV virus accumulated in roots, tendrils, leaves and pods, accompanying PoPDS (phytoene desaturase) silencing. No other obvious symptoms were induced by ALSV in *P. sativum*. This is also the first report of direct agro-mediated stem inoculation by ALSV VIGS vector in legumes species. ALSV vectors can be used for high-throughput gene function analysis and for testing the susceptibility of different genotypes by agro-injection.

## 2 | MATERIALS AND METHODS

### 2.1 | Plant material and growth conditions

Seeds of field pea (*P. sativum*) cultivars were obtained from Dr Dengjin Bing (Agriculture and Agri-Food Canada; AAC Lacombe, AAC Carver, and AAC Comfort; Bing, Beauchesne, McLaren, Gan, & Cuthbert, 2018a, 2018b; Bing, Beauchesne, McLaren, Vera, & Gehl, 2014) and Dr Tom Warkentin (University of Saskatchewan; CDC Golden and CDC Meadow; Warkentin, Vandenberg, Banniza, & Slinkard, 2004; Warkentin, Vandenberg, Tar'an, Barlow, & Ife, 2007). Seeds of *P. vulgaris* cv. Black Valentine, a vintage cultivar, OAC Rex (Michaels, Smith, Larsen, Beattie, & Pauls, 2006), *N. benthamiana*, *C. quinoa*, and *G. max* cv. Williams B2 (Bernard & Cremeens, 1988) were used for testing. All seeds were planted in Pro Mix BX Mycorrhizae (Premier Tech, Rivière-du-Loup, Québec), and all plants (inoculated or non-inoculated) were grown in a growth cabinet (Environmental Growth Chambers, Chagrin Falls, OH) at 22°C under a 16-hr light/8-h dark cycle. The ALSV isolates were obtained under a Materials Transfer Agreement between Iwate University and Agriculture and Agri-Food Canada, and an import permit granted by the Canadian Food Inspection Agency (P-2013-02404). All material was handled with the requirements of Plant pest containment level 1 as described in the Containment Standards for Facilities Handling Plant Pests (plant compliance number: PC-2013-032).
2.2 | Viral vectors

pBICAL1, pBICAL2, and pBICAL2-NbPDS were kindly provided by Dr Masanori Kaido, Kyoto University, Japan (Kawai et al., 2014). Following the method of Kawai et al. (2014), we subcloned the ALSV RNA 1 from pEALSR1 (Li et al., 2004) into pCAMBIA1300 using primers Sall/pUC18-F and Kpnl/nos-R, which include Sall and Kpnl restriction enzyme sites, respectively (Table 1). The polymerase chain reaction (PCR) product was digested with Sall and Kpnl and inserted into the same sites of pCAMBIA1300, generating pCALSV1 (Figure 1). For ALSV RNA2 vector construction, a 300-bp PvPDS (Phaseolus vulgaris phytoene desaturase) fragment was amplified from P. vulgaris cv. OAC Rex cDNA using primer pair ORexPDSVIGS280F and ORexPDSVIGS562R having Xhol and BamHI sites, respectively (Table 1). The PvPDS PCR product was cloned using the Zero Blunt TOPO PCR cloning kit (Thermo Fisher Scientific, https://www.thermoscientific.com), sequenced, and a positive clone was digested with Xhol and BamHI-SpeI fragment containing pEALS2R-PvPDS middle part was cloned into pCALSV2-1/3 digested with the same restriction enzymes to generate pCALSV2-PvPDS (Figure 1). The experimentally determined sequences of pCALSV1 and pCALSV2-PvPDS are provided as supporting information (Data S1).

2.3 | Agro-mediated inoculation

For Agro-mediated inoculation, pCALSV1 and pCALSV2-PvPDS plasmids were transformed into Agrobacterium strain EHA105. pBICAL1, pBICAL2, and pBICAL2-NbPDS plasmids were also transformed into EHA105. The 35S-P19 vector for the expression of the P19 viral suppressor from tomato bushy stunt virus in Agrobacterium strain C58C1 was used in all transformations (Lakatos, Szittya, Silhavy, & Burgván, 2004; Silhavy et al., 2002). All strains were grown in LB-medium containing 100-μg/ml kanamycin and 25-μg/ml rifampicin at 28°C. Cells were pelleted and washed twice with sterile water and resuspended in agroinduction buffer [10-mM MgCl2, 10-mM 2-(N-morpholino)ethanesulfonic acid (pH 5.7), 100-μM acetosyringone] to a final OD600 of 1.0. The cell suspension was incubated for 3 hr at room temperature and then mixed 1:1:1 (RNA1: RNA2: 35S-P19) for N. benthamiana infiltration or injection into 7- to 10-day-old legume seedling stem using a 1-ml syringe with a 26-gauge needle. Three to five points were injected per plant. The plants were then sprayed with water to retain humidity without covering in growth cabinet.

2.4 | Mechanical inoculation

To test the infection of *P. sativum*, *P. vulgaris*, and *G. max* by ALSV using mechanical inoculation, infected pea, *N. benthamiana*, or *C. quinoa* upper leaves were ground in liquid nitrogen with extraction buffer (0.1-M Tris-HCl, pH 7.8, 0.1-M NaCl, 5-mM MgCl2) and inoculated to the adaxial surface of two carborundum dusted primary leaves from 15- to 20-day-old plants.

| Oligonucleotide name | Sequence (5’ – 3’) |
|----------------------|-------------------|
| Sall/PUC18-F         | CACACA GTGCAC GTTTTCTCCAGTCACGAGTTG |
| Kpnl/Nos-R           | CACACA GTGACC GATCTGAAATACATAGTGACACCC |
| ORexPDSVIGS280F      | TACATCTGAGTCTGCGCTGGCTACAAC |
| ORexPDSVIGS562R      | TACATGGATCTGGTTATTGCGTATGGT |
| PsPDS-Q-F            | CAGGAATGCCCTGCTTTCATAC |
| PsPDS-Q-R            | CAGGAATGCCCTGCTTTCATAC |
| PsActin-F            | CACATTTGGCCGTGAAAGAT |
| PsActin-R            | TAATAGTATATTAATGATGATCATGGAT |
| CT55                 | GCATCTTGGCCCAAGAC |
| CT56                 | TGCAAGCCAGGCCAGATAG |
| CT57                 | AGGACCTTATGTGGGGTT |
| CT58                 | GTGGAATCTCTCTCAATAC |
| CT93                 | AAATCTCTTGCTTCTTCAATGAGGT |
| CT94                 | ATAAAAAGAAGACCTTAGAA |

Note. Restriction sites are highlighted in italics.
RESULTS

3.1 | Agroinfiltration of infectious ALSV clone in *N. benthamiana* and legume species

Agroinfiltration of VIGS vectors is popular and extremely efficient in some species but not in legumes. Agro-infiltration of ALSV infectious clones has been carried out on *N. benthamiana* with 100% efficiency (Kon & Yoshikawa, 2014). We conducted leaf infiltration with *Agrobacterium* (EHA105) containing pCALSV1, pCALSV2-PvPDS (and for *N. benthamiana*, also with pBI62-NbPDS) and 35S-P19 on *N. benthamiana* and three legume species, *P. sativum* cv. AAC Comfort, *P. vulgaris* cv. Black Valentine and OAC Rex, and *G. max* cv. Williams 82. The abaxial surfaces of cotyledons and primary leaves of 10-day-old *G. max* cv. Williams 82 seedlings were infiltrated with Agrobacterium solution (Lim et al., 2016). Two primary leaves of 10-day-old *P. sativum* and 7-day-old *P. vulgaris* were infiltrated. Not surprisingly, obvious photobleaching symptoms appeared on *N. benthamiana* 14 dpi with pBI62-NbPDS (Figure 2c). The infection efficiency was 100%, the same as in previous results. Very light photobleaching occurred on *N. benthamiana* at 14 dpi inoculated with pCALSV2-PvPDS (Figure 2b). Using RNA1-specific primer pair CT57 and CT58 and RNA2-specific primer pair CT93 and CT94, virus was detected from both plants by RT-PCR (Figure 2). However, none of the infiltrated pea plants [cv. AAC Comfort (0/10)], common bean plants [cv. Black Valentine (0/13), OAC Rex (0/12)], or soybean plants [cv. Williams B2 (0/12)] showed symptoms of PDS silencing, and no virus was detected by RT-PCR from these plants at 30 dpi (data not shown).

3.2 | *Agrobacterium* stem injection inoculation with infectious ALSV clone in three legume species

As an alternative to leaf infiltration of *Agrobacterium* containing ALSV, we assessed direct stem injection. Seven-day-old seedlings of *P. sativum* cv. AAC Comfort, *P. vulgaris* cv. Black Valentine and OAC Rex, and *G. max* cv. Williams 82 were injected into stems with *Agrobacterium* containing ALSV VIGS vectors (pCALSV1 and pCALSV2-PvPDS) and 35S-P19. Three to five points were injected with approximately 50-μl mixed solution per point in each seedling. Water was sprayed to retain humidity. Photobleaching symptoms (Figure 3b) started showing after 23 dpi on pea. No silencing symptoms appeared on common bean or soybean (data not shown).

We monitored the remainder of the life cycle of the pea plants infected by ALSV. Sepals began to bleach at approximately 35 dpi and pods first showed symptoms from approximately 45 dpi (Figure 3d). The typical virus infection movement process was seen for virus systemic movement through the phloem with photobleaching in leaves extending from the leaf veins (Figure 3c). Symptoms continued for up to 2 months until seeds were harvested. Pods and seeds were generally visually smaller with lower yields than uninfected plants, presumably because of the decrease in photosynthesis (Figure 3e).

3.3 | Susceptibility to ALSV of five *P. sativum* cultivars

Five pea cultivars (AAC Carver, AAC Comfort, AAC Lacombe, CDC Meadow, and CDC Golden) were tested for ALSV-derived gene silencing. Pea plants were stem injected with *Agrobacterium* containing pCALSV1, pCALSV2-PvPDS, and 35S-P19, and at 23 dpi, photobleaching symptoms appeared on the inoculated leaves and stems in all five genotypes, demonstrating both virus assembly and systemic movement. At 30 dpi, infection rates of 7.6% to 36.3% were obtained (Table 2). Cultivar Lacombe was the most susceptible genotype in this initial test and subsequently, cv. Lacombe was chosen for further mechanical inoculations. Using RT-PCR, both RNA 1 and RNA 2 of ALSV were detected in systemic tissues (data not shown).

3.4 | Mechanical inoculation from infected pea sap

Mechanical inoculation was carried out using *N. benthamiana* sap infected with pCALSV1, pCALSV2-PvPDS, and 35S-P19 on *N. benthamiana* and *C. quinoa*. Symptoms on *C. quinoa* appeared at approximately 14 dpi (Figure 4a,b), and virus was detected by RT-PCR (Figure 4c). Infected *N. benthamiana* or *C. quinoa* sap was used to mechanically inoculate *P. sativum*, *P. vulgaris*, and *G. max*. One month later, no plants showed photobleaching symptoms, and no virus was detected by RT-PCR. However, photobleached pea leaves from plants that had been stem injected were collected, ground with extraction buffer and the two bottom leaves of 12-day-old pea plants (cv. AAC Lacombe) were dusted with carborundum and rubbed with infected...
pea leaf sap. At 23 dpi, photobleaching symptoms appeared on the mechanically inoculated P. sativum. The infection rate exceeded 80% on P. sativum (8 out of 10, 9 out of 10, and 9 out of 11, cv. AAC Lacombe) but 0% on P. vulgaris and G. max inoculated with infected pea sap.

3.5 Virus accumulation and PDS expression level in different organs

Virus could be detected from leaves, tendrils, roots, sepals, petals, carpels, pods, and seed coat by RT-PCR (Figures 5a and 6). Virus accumulation and PDS expression level were monitored in different organs using RT-PCR. Virus RNA1 and RNA2 were detected in leaves, tendrils, roots, sepals, petals, carpels, pods, and seed coat by RT-PCR (Figures 5a and 6).
detected in the leaves, tendrils, and roots was accompanied by PDS transcript levels decreasing in these tissues (Figure 5). All the tissues from flowers and developing seeds exhibited virus infection except for the developing embryo (Figure 6a). Low titer of virus was found in seed coats, as determined by real-time RT-PCR (Figure 6b). Endpoint RT-PCR confirmed the positive results for seed coats (Figure 6a).

4 | DISCUSSION

*Pisum sativum* is an economically important legume crop, and an International Consortium has been formed to tackle sequencing of the pea genome (Alves-Carvalho et al., 2015; Gali et al., 2019; Tayeh et al., 2015). VIGS has the potential to be an extremely useful tool, complementing these efforts. The selection of a virus as a VIGS vector depends on its ability to infect and propagate within and between plant cells (Vaghchhipawala et al., 2011). ALSV has a broad host range and could be used to silence genes in many plant species. However, it has been necessary to use infected leaves of *N. benthamiana* or quinoa as a source of concentrated ALSV, requiring one or two cycles of propagation (Igarashi et al., 2009; Satoh et al., 2014) or RNA extraction for microparticle bombardment (Yamagishi & Yoshikawa, 2009) to infect legume plants. Here, we developed a convenient Agrobacterium stem injection method for delivery of ALSV vectors into *P. sativum* plants. Entering the phloem vasculature directly by stem injection could help the virus move to systemic leaves effectively. Pea plants were infected with ALSV vectors and uniform silencing symptoms were obtained with *PvPDS*. The infection rate of the agro-injection method was up to 36.3% in one of the *P. sativum* cultivars, AAC-Lacombe. Secondary mechanical inoculation using pea sap was a highly effective tool, with over 80% infection rate, for silencing of endogenous genes. Gedling et al. (2018) reported that the soybean genotype used in the present study, Williams 82, is not susceptible to ALSV infection. Similarly, common bean was previously reported to

### TABLE 2

| Cultivar          | Number of infected plants/inoculated plants | Average (%) ± SD |
|-------------------|---------------------------------------------|------------------|
| AAC Carver        | 1/18 (5%); 1/10 (10%); 1/14 (7.1%)          | 7.6 ± 2.3%       |
| AAC Comfort       | 5/17 (29.4%); 5/13 (38.5%); 6/17 (35.3%)    | 34.4 ± 4.6%      |
| AAC Lacombe       | 6/17 (35.3%); 8/21 (38.1%); 5/13 (38.5%)    | 36.3 ± 1.8%      |
| CDC Meadow        | 3/14 (21.4%); 3/12 (25%); 2/11 (18.2%)      | 21.5 ± 3.4%      |
| CDC Golden        | 1/14 (7.1%); 1/11 (9.1%); 1/12 (8.3%)       | 8.2 ± 1.0%       |

### FIGURE 4

Symptoms of ALSV infection in *Chenopodium quinoa* plants mechanically inoculated using *Nicotiana benthamiana* sap. (a) pCALSV1, pCALSV2-PvPDS, and 35S-P19; (b) buffer control; (c) reverse transcription polymerase chain reaction to detect virus RNA1 and RNA2
be outside the host range of ALSV, as tested with cv. Hatumodori No. 2 (Yoshikawa et al., personal communication).

A high efficiency of mechanical inoculation on pea plants for secondary inoculations using pea leaf sap was demonstrated in this report. The sap from infected \textit{N. benthamiana} or \textit{C. quinoa} leaves also was tested to inoculate legume species, and no infection was found on pea plants. The sap from quinoa plants was successfully used to infect pea; however, this required two cycles of propagation in the quinoa plants (Igarashi et al., 2009). We have not investigated why sap from infected pea leaves was more effective than virus sourced from tobacco or quinoa. However, a high enough titer of ALSV inocula seems necessary for further multiplication of the virus and initiation of VIGS in a new cell. In the work by Satoh et al. (2014) in which ALSV was used as a vaccine in pea, inocula were concentrated by precipitation using a bentonite solution and PEG-6000 from \textit{N. benthamiana} leaf homogenates, increasing efficacy, which emphasizes that a high ALSV titre is required for pea infection. This procedure was not used in the present study. It is also well known that RNA viruses evolve relatively fast and can adapt rapidly to a new host, and this may have contributed to the higher efficiency observed with secondary pea infections. The mutation rate of a typical virus-encoded RNA-dependent RNA polymerase is $10^{-4}$ to $10^{-6}$ errors per nucleotide.

Following inoculation, ALSV virus could be detected in multiple pea organs (leaves, roots, tendrils, sepals, petals, and seed coats). Using this approach, it appears that no virus was transmitted to the developing seeds. However, VIGS of a soyPDS gene was maintained in the next generation plants by the seed transmission of ALSV-soyPDS (Yamagishi & Yoshikawa, 2009). The ability of seed transmission might be species-specific. Nevertheless, an alternate protocol, inoculating pea plants closer to the timing of flower development might produce different results. Moreover, the presence of the virus in the seed coat and not in the developing embryo may have interesting applications for studying nutrient partitioning. There is doubtless room to improve the efficacy of the ALSV-pea VIGS system, but we see it currently as a promising tool for pea functional genomics. An obvious application is gene inactivation as in the present study. Additional potential uses include genome editing, metabolic engineering, particularly of flavor determinants, and targeting of epigenetic modifications at specific genomic loci (Kon & Yoshikawa, 2014; Pasin, Menzel, & Daròs, 2019).

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**AUTHOR CONTRIBUTIONS**

RX, FM, and CT contributed to conceptualization; RX and AP to investigation; NY and AW contributed resources; RX contributed to writing--original draft; and FM and CT contributed to writing--review and editing.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.
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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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