Planta (1997) 203: 327–331

Planta
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Loss of callose in the stigma papillae does not affect the *Brassica* self-incompatibility phenotype

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Received: 14 March 1997 / Accepted: 15 April 1997

Abstract. As part of the *Brassicaceae* self-incompatibility response, callose is deposited in the stigma papillar cells. To determine if callose plays an important role in the rejection of incompatible pollen by the stigma, transgenic *Brassica napus*. L. plants were produced which express the tobacco β-1,3-glucanase cDNA (the enzyme which degrades callose) in the stigma papillae. Using aniline blue fluorescence, little or no callose was detected in the papillar cells of transgenic stigmas. However, the self-incompatibility system appeared to be unaffected based on the lack of pollen tube growth and the subsequent lack of seed set. The transgene had no effect on compatible pollinations. Thus, while callose deposition is associated with the *B. napus* self-incompatibility response, it is not required for the rejection of incompatible pollen.

Key words: *Brassica* (callose) – Callose – Self-incompatibility response – Pollen – Stigma

Introduction

In the *Brassica* family, self-incompatibility is a mechanism used by the plant to promote outbreeding. The system is under the control of a single multi-allelic genetic locus called the *S*-locus which prevents fertilization between plants sharing a common *S*-allele (Bateman 1955). The *S*-locus has been shown to encode at least two transcribed genes, the *S-locus glycoprotein* (*SLG*) and the *S-locus receptor kinase* (*SRK*) (Nasrallah et al. 1985; Stein et al. 1991). The *SLG* gene encodes a secreted glycoprotein that accumulates in the cell wall of the stigma papillae cells during anthesis (Kandasamy et al. 1989), while the *SRK* gene encodes a membrane-associated receptor kinase with serine and threonine autophosphorylation activity (Goring and Rothstein 1992; Stein and Nasrallah 1993; Delorme et al. 1995; Stein et al. 1996). From what is known about the activation of receptor tyrosine kinases in animal systems (Ullrich and Schlessinger 1990), it is thought that a ligand on the surface of the pollen may bind to the extracellular domain of the *SRK* resulting in the activation of the kinase domain. This activated kinase domain could then act upon downstream targets in the stigma papillae, mediating a signalling cascade leading to pollen rejection (Nasrallah and Nasrallah 1993).

Pollen rejection is marked by little or no pollen hydration, failure of pollen tube growth to extensively penetrate the stigma and the rapid deposition of callose, a β-1,3-glucan, in the stigma papillae cells in direct contact with pollen grains (Knox 1973; Roberts et al. 1980; Kerhoas et al. 1983). The callose response had long been reported as a phenomenon exclusive to self-incompatible pollinations with little or no callose being deposited in self-compatible pollination or in stigma papillae cells not in direct contact with incompatible pollen (Knox 1973; Dumas and Knox 1983; Kerhoas et al. 1983). The strong correlation of the callose response with the presence of incompatible pollen had led to the suggestion that this laying down of callose "plugs" may have a specific role in the sporophytic self-incompatibility response and may play an active role in pollen inhibition (Dumas and Knox 1983). Callose is also observed in the growing tips of pollen tubes. It is a general phenomenon of many plant species and unrelated to self-incompatibility (Fosket 1994).

It is of great interest to fully elucidate the role that callose may play in pollen rejection at the stigma. In order to determine if callose has any role in the self-incompatibility response, transgenic *Brassica napus* plants were constructed that overexpress a β-1,3-glucanase in their stigma papillae cells. This enzyme would specifically degrade any callose and prevent the accumulation of callose plugs in the stigma. Targeted β-1,3-glucanase activity has been successfully used in transgenic tobacco to degrade the microsporocyte callose wall in anther tissue (Worrall et al. 1992). Our results indicate that callose formation can be effectively

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blocked in the stigmas of these transgenic *B. napus* plants with no apparent effects on the self-incompatibility signalling response and subsequent pollen rejection.

Materials and methods

Production of Brassica napus plants transformed with the β-1,3-glucanase transgene. The tobacco β-1,3-glucanase cDNA was polymerase chain reaction (PCR) amplified from first-strand cDNA using two β-1,3-glucanase-specific primers as described by Worrall et al. (1992). Ten micrograms of total tobacco leaf RNA was used in a first-strand cDNA reaction primed with oligo dTprimer. The cDNA was then PCR-amplified with the β-1,3-glucanase specific primers for 15 amplification cycles consisting of 45 s at 94 °C, 45 s at 55 °C and 1 min and 10 s at 72 °C. This was followed with 15 amplification cycles consisting of 45 s at 94 °C, 45 s at 65 °C and 1 min and 10 s at 72 °C. The PCR reaction was then diluted fourfold and two amplification cycles consisting of 5 min at 72 °C were used. The 1018-bp PCR product was gel-purified, digested with *Xba* I and *Sac* II, subcloned into the *Xba* I/*Sac* II sites of pBScII, and sequenced. The PCR clone was found to have an identical sequence to the published β-1,3-glucanase gene (Ohme-Takagi and Shinshi 1990).

To construct the plant transformation vector, the *Xba* I/*Sac* II β-1,3-glucanase fragment was blunt-ended with Klenow and cloned in between the SLR1 promoter and the nos terminator at a *Sma* I site. The binary vector was introduced into *Agrobacterium* strain LBA4404 (Hoekema et al. 1983) and self-incompatible *Brassica napus* W1 plants were then transformed via *Agrobacterium* mediated transformation as described by Moloney et al. (1989).

Isolation and analysis of DNA. Isolation and purification of plant DNA was carried out as previously described (Goring et al. 1992). Approximately 5 μg of genomic DNA was digested with *Hind* III and loaded on a 1% agarose gel. After gel electrophoresis overnight, the DNA was transferred to a Zetaprobe membrane and hybridized with the α-^{32}P-labelled β-1,3-glucanase cDNA overnight at 42 °C as previously described (Goring et al. 1992). The blot was washed at 65 °C with a final stringency of 0.1 × SSC and 0.1% (w/v) SDS and exposed to X-ray film. The membrane was then stripped and hybridized to the 1.5-kb SLG-910 cDNA.

Isolation and analysis of RNA. Total RNA was isolated from pistils (large buds and freshly opened flowers) as previously described (Goring et al. 1992). Polyadenylated RNA was isolated using a polyA Spin mRNA Isolation Kit (New England Biolabs), separated on a 1% RNA formaldehyde gel, transferred to a nylon membrane, and hybridized with radiolabeled DNA probes used in genomic DNA analysis under the same conditions. The RNA blot was first hybridized with the 1.0-kb β-1,3-glucanase cDNA, and then with the 1.5-kb SLG-910 cDNA.

Aniline blue microscopy of callose formation during pollination. All male tissues and petals were removed from buds 1 d prior to anthesis. The remaining stigmas were brushed lightly with anthers of either W1, Westar or the transgenic plants and bagged to prevent contaminating pollen grains from landing on the stigma. All pollinations were allowed to proceed overnight. The next day, pistils were removed and fixed in 3:1 (u/v) ethanol:glacial acetic acid solution for at least 15 min. After rinsing with water, the pistils were treated with 1 N NaOH at 60 °C for 1 h, and then rinsed twice with water. A solution of 0.1% aniline blue in 0.1 M K_3PO_4 (pH 7.0) buffer was added and the pistils were then immediately wet-mounted on slides and examined using normal and UV fluorescence microscopy.

Seed setting of β-1,3-glucanase transgenic plants. Buds 1 d prior to anthesis were stripped of all male tissue and pollinated with either W1, Westar or transgenic pollen, bagged for one week and then left uncovered for the resulting pods to mature. Pods were collected and number of seeds set were recorded for each pollination type.

Results

Production of self-incompatible Brassica napus transformed with the β-1,3-glucanase cDNA. To investigate the role of callose in the *Brassica* self-incompatibility response, we transformed a self-incompatible *Brassica napus* line called W1 (Goring et al. 1992). The W1 line was produced by introgressing a *Brassica campestris* *S*-allele into the self-compatible *B. napus* cultivar, Westar (Goring et al. 1992). Thus, W1 serves as a control for self-incompatible pollinations and Westar as the control for self-compatible pollinations. Expression of the tobacco β-1,3-glucanase cDNA which included the N-terminal signal peptide but lacked the C-terminal propeptide (CTTP), was targeted to the stigma by linking it with the tissue-specific *S*-Locus Related (*SLR*) 1 promoter (Franklin et al. 1996). The *SLR1* gene is expressed at high levels in the *Brassica* stigma papillae prior to anthesis (Lalonde et al. 1989).

From two original lines of transgenic plants, five plants (second generation) were analysed further: one from first line (Glc-1) and four from the second line (Glc-2a, b, c, d).

Analysis of the DNA of β-1,3-glucanase transgenic plants. To confirm the presence of the β-1,3-glucanase cDNA and to examine copy number, DNA blot analysis was performed. Genomic DNA samples were isolated from the transgenic plants, W1 and Westar, digested with *Hind* III which cuts once in the transformation vector,

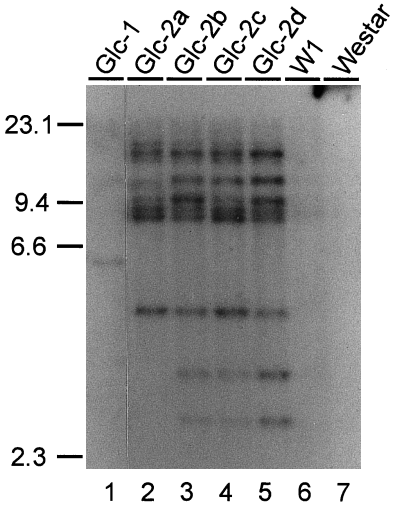

Fig. 1. DNA blot analysis of the β-1,3-glucanase transgenic *Brassica* plants. Genomic DNA samples from five transgenic plants, self-incompatible W1 and self-compatible Westar were digested with *Hind* III and hybridized to the β-1,3-glucanase cDNA. There is one *Hind* III site in the β-1,3-glucanase transformation vector. For Glc-1, a single band of approximately 6.2 kb indicates the presence of a single copy of the transgene (*lane 1*), while the other four transgenic plants carry multiple copies (*lanes 2–5*). As expected, the untransformed W1 and Westar DNA do not hybridize to the tobacco β-1,3-glucanase cDNA

and hybridized to the β-1,3-glucanase cDNA (Fig. 1). Glc-1 carries a single insertion which is seen as a 6-kb band when digested with *Hind* III, (Fig. 1, lane 1), while Glc-2 plants carry multiple copies of the transgene (Fig. 1, lanes 2–5). The four Glc-2 plants also show some differences in the banding pattern, indicating that some of the copies are integrated at different sites or were lost from one generation to the next (Fig. 1, lanes 2–5). The tobacco β-1,3-glucanase cDNA did not cross-hybridize to any endogenous β-1,3-glucanase genes in W1 and Westar (Fig. 1, lanes 6 and 7). The genomic DNA samples were also hybridized to the SLG-910 cDNA (Goring et al. 1992) to confirm the presence of the W1 *S*-locus (data not shown).

Analysis of the RNA of β-1,3-glucanase transgenic plants. Expression levels of the β-1,3-glucanase cDNA were examined by RNA blot analysis of poly(A)$^+$ pistil RNA from the five transgenic plants (Fig. 2). The highest levels of steady-state RNA were detected in Glc-1 (Fig. 2, lane 1). The four Glc-2 plants showed variations in the β-1,3-glucanase RNA levels, with Glc-2c and Glc-2d showing higher levels than Glc-2a and Glc-2b (Fig. 2, lanes 2–5). Only a very faint signal could be detected for Glc-2a and Glc-2b (Fig. 2, lanes 3 and 4). However, for all plants, the expression levels were sufficient to have an effect on callose deposition (see below). Hybridization of the same filter with the SLG-910 cDNA showed similar levels of this message in all four lanes. Using reverse transcriptase-PCR, the presence of the β-1,3-glucanase transcripts in all transgenic plants was verified (data not shown).

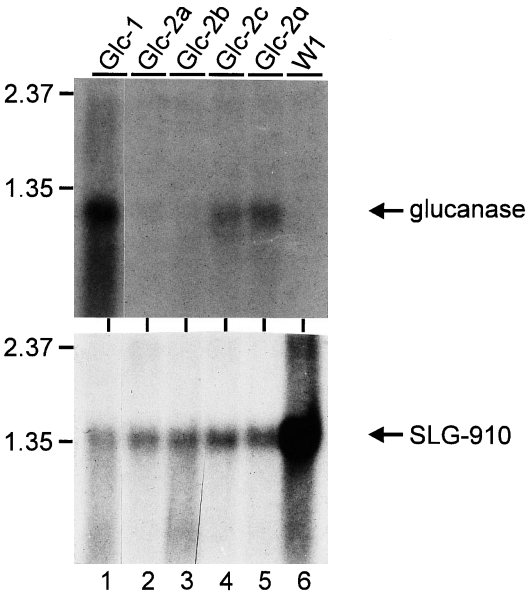

Fig. 2. RNA blot analysis of the β-1,3-glucanase transgenic *Brassica* plants. Polyadenylated RNA from the five transgenic plants and W1 was hybridized with the β-1,3-glucanase cDNA and the SLG-910 cDNA. β-1,3-Glucanase mRNA (*top panel*) could be detected for Glc-1 (*lane 1*) and the Glc-2 transgenic plants (*lanes 2–5*), though for Glc-2a and Glc-2b only a very faint band was detected (*lanes 2, 3*). The SLG-910 cDNA (*bottom panel*) was used as a probe to check message integrity, and SLG-910 mRNA could be detected for allsamples. The very strong signal for W1 is likely due to a higher amount of RNA in the lane as well as W1 being a homozygous plant while the transgenic plants appear to be heterozygous for the SLG-910 gene

Callose deposition during self-incompatible pollinations. To examine callose deposition during pollinations, different pollinations were carried out between W1, Westar, and the transgenic plants. Callose was detected by using aniline-blue fluorescence staining (Kho and Baer 1968). Self-pollination of the self-incompatible W1 resulted in callose deposition as detected by the bright fluorescence in the W1 papillae (Fig. 3B). However, similar pollinations of the five transgenic plants with W1 pollen resulted in little or no callose deposition (Fig. 3D,F). Examples of a Glc-1 (higher β-1,3-glucanase mRNA levels) pollination and a Glc-2a (lower β-1,3-glucanase mRNA levels) pollination demonstrate that no fluorescence could be detected for Glc-1 (Fig. 3D), while only very low levels of fluorescence could be detected for Glc-2a (Fig. 3F). In addition, as expected for self-incompatible pollinations, little or no germinating pollen could be detected (Fig. 3B,D,F; and Fig. 4F).

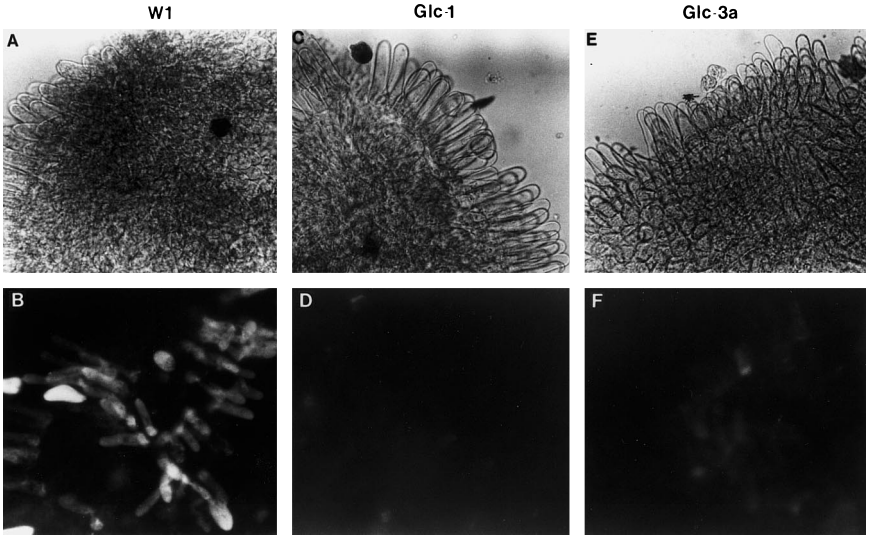

Fig. 3A–F. Callose formation in transgenic *Brassica* plants during W1 pollination. **A, B** Bright field (**A**) and UV fluorescence (**B**) images of a self-incompatible pollination on a W1 stigma. Callose deposition in the W1 stigma papillae cells results from contact with a self-incompatible pollen. **C, D** Bright field (**C**) and UV fluorescence (**D**) images of a Glc-1 pistil pollinated with W1 pollen. In the Glc-1 stigma, callose is not detected as a result of the β-1,3-glucanase transgene. **E, F** Bright field (**E**) and UV fluorescence (**F**) images of a Glc-2a pistil pollinated with W1 pollen. Glc-2a shows significantly reduced levels of callose formation compared to the self-pollinated W1 pistils

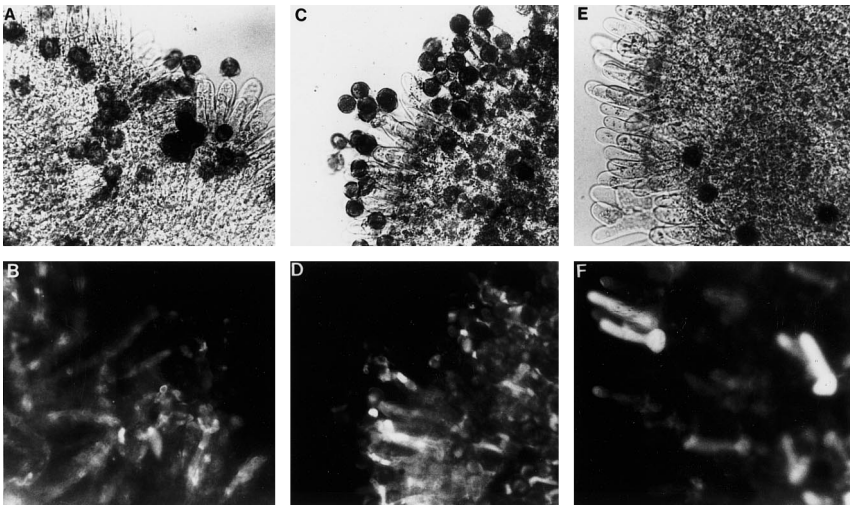

Fig. 4A–F. Callose formation in self-compatible and self-incompatible pollinations. **A, B** Bright field (**A**) and UV fluorescence (**B**) images of a self-compatible pollination on a Westar stigma with Glc-1 pollen. Callose deposition is apparent in the pollen tubes emerging from germinated Glc-1 pollen grains. **C, D** Bright field (**C**) and UV fluorescence (**D**) images of a Glc-2a pistil pollinated with Westar are typical of a self-compatible pollination, as Westar pollen grains have germinated and the pollen tubes stained with callose are growing down the stigma cells. **E, F** Bright field (**E**) and UV fluorescence (**F**) images of a self-incompatible pollination with a W1 pistil and Glc-1 pollen show that pollen grains failed to germinate and grow pollen tubes typical of a self-incompatible response

Compatible pollinations between Westar and the transgenic plants resulted in normal pollen germination on all plants. Examples of compatible pollinations between Glc plants and Westar are shown (Fig. 4B, D). Callose deposition in the pollen tube is unaffected by the β-1,3-glucanase transgene which, as expected, has activity only in the stigma papillae cells. When the transgenic pollen was placed on W1 pistils, normal callose deposition was detected in the incompatible W1 papillae (Figure 4F, an example with Glc-1 pollen).

Effect of the β-1,3-glucanase transgene on self-incompatibility and fertility. The initial pollination experiments suggested that the β-1,3-glucanase transgenic plants retained their self-incompatibility phenotype despite the absence of callose and that compatible pollinations were not affected. To confirm this, different pollinations were performed between W1, Westar and three of the transgenic plants. Seeds were allowed to develop and were then counted (Table 1). The control compatible pollination of Westar resulted in a number of seeds set per pod ranging from 16 to 24 while the control self-incompatible W1 pollination resulted in 0 seeds per pod (Table 1, A and B). Cross-compatible pollinations between Westar and the transgenic plants resulted in seeds set ranging from 11 to 26 seeds per pod (Table 1, C). Thus, the transgenic plants are fully compatible with Westar. Cross-incompatible pollinations between W1 and the transgenic plants resulted in 0 to 2 seeds per pod (Table 1, E) indicating that the self-incompatibility system is fully functional despite the removal of callose from the self-incompatibility response.

Table 1. Numbers of seed set from *Brassica* pollinations. A) Self-compatible pollination of Westar pistils with Westar pollen. B) Self-incompatible pollination of W1 pistils with W1 pollen. C) Westar pollen on Glc-1, Glc-2a and Glc-2b pistils. D) Glc-1, Glc-2a or Glc-2b pollen on Westar pistils. E) W1 pollen on Glc-1, Glc-2a and Glc-2b pistils. F) Glc-1, Glc-2a or Glc-2b pollen on W1 pistils

	Pollination event Pollen	Pistil	Number of seeds per pod
A)	Westar (Self-incompatible)	Westar	16, 18, 20, 24
B)	W1 (Self-incompatible)	W1	0, 0, 0, 0
C)	Westar Westar Westar	Glc-1 Glc-2a Glc-2b	11, 15, 15, 16 24, 25, 26 22, 23, 25
D)	Glc-1 Glc-2a Glc-2b	Westar Westar Westar	20, 20, 23, 24 23, 24, 28 26, 27
E)	W1 W1 W1	Glc-1 Glc-2a Glc-2b	0, 0, 0, 0, 0 0, 0, 2 0, 0, 1
F)	Glc-1 Glc-2a Glc-2b	W1 W1 W1	0, 0, 0 0, 1, 1 0, 1, 1

Discussion

The molecular mechanism underlying self-incompatibility, which prevents pollen hydration or pollen tube penetration of the stigma is unknown. As callose has been proposed to play a role in the self-incompatibility response (Dumas and Knox 1983), we have created transgenic plants which produce β-1,3-glucanase, the enzyme which degrades callose, in the stigma. The analysis of these transgenic plants revealed little or no detectable levels of callose after incompatible pollinations with either W1 or self pollen, along with the failure of the incompatible pollen to germinate. Compatible cross-pollinations between Westar and the transgenic plants resulted in the germination of Westar and transgenic pollen grains, and pollen tubes were observed to grow down into the style. Transgenic pollen when placed on W1 were rejected, indicating that the transgenic pollen is phenotypically identical to W1 pollen and unaffected by the β-1,3-glucanase transgene. Seed set data also confirmed that the self-incompatibility system was intact, and that the β-1,3-glucanase transgene did not affect the fertility of the transgenic plants. Taken

together, our results indicate that the self-incompatibility system is unaffected by the lack of callose deposition in the stigma cells. Thus, while callose deposition seems to occur in parallel with the self-incompatibility response, it does not play an essential role in pollen rejection.

Other research on the role of callose in the *Brassica* self-incompatibility response has tested the use of chemical inhibitors on pollen germination (Singh and Paolillo 1990; Elleman and Dickinson 1996). With the pretreatment of pistils with 2-deoxy-D-glucose, Elleman and Dickinson (1996) saw a partial reduction of callose deposition while Singh and Paolillo (1990) reported that 2-deoxy-D-glucose resulted in a total absence of callose though no data were shown. In addition, callose deposition was found to be a calcium-dependent process and calcium inhibitors were found to reduce or abolish callose deposition (Singh and Paolillo 1990). In these studies, the self-incompatibility response did not appear to be affected as there was no germination of incompatible pollen. The advantage of the system that we have developed over these studies is that the pollinations are done directly on the plant and do not require removal of the pistil. Thus, pollinated pistils can be left until seed-set data are obtained and the effectiveness of the self-incompatibility response to prevent fertilization in the absence of callose can be truly evaluated. In addition, any potential side effects of chemical inhibitors are avoided by this approach. Callose formation can be induced in the stigma through direct touch or contact with foreign particles, wounding, and pathogens (Aist 1976; Grotha 1986). The callose response during a self-incompatible pollination may be due to one of these factors. Alternatively, a compatible pollination may activate some mechanism that prevents callose synthesis, though even with compatible pollinations some callose can be detected.

In conclusion, the abolition of callose by the expression of a β-1,3-glucanase cDNA in the stigma papillae cells of these transgenic plants has no inhibiting effects on the self-incompatibility system of *Brassica* and allows these plants to fully retain their fertility.

We thank John Dearnaley for assistance with the microscopy. We are also very grateful for the SLR1 promoter provided by Martin Trick (John Innes Centre). This work supported by grants from the Natural Sciences and Engineering Research Council of Canada to D.R.G. and S.J.R.

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