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Detection of Trace Level of *cp4epsps* Transgene on Sclerotia of *Sclerotinia sclerotiorum* Formed in Diseased Plants of Roundup Ready® Canola (*Brassica napus*)

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ABSTRACT

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The area seeded to genetically modified crops has been steadily increasing since their introduction in the early 1990s. However, there have been concerns relating to the persistence and stability of transgenic DNA from genetically modified crops and subsequent outflow into the environment. The *cp4 5-enolpyruvylshikimate-3-phosphate synthase* (*cp4epsps*) gene confers glyphosate resistance in Roundup Ready® (RR) canola (*Brassica napus* event RT73). *Sclerotinia sclerotiorum* is an important pathogen causing stem blight and pod rot of canola. The aim of this study was to detect *cp4epsps* transgenic DNA on sclerotia of *Sclerotinia sclerotiorum* formed in diseased stems of RR canola grown in the field. In this 2-year study, sclerotia collected from naturally infected stems of RR canola and conventional canola at the time harvest were screened for the presence of 1363 bp *cp4epsps* and smaller transgene fragments by PCR (limit of detection 50 pg) and Southern hybridization (limit of detection < 5 pg). The complete *cp4epsps* transgene could not be detected in any of the analyzed sclerotia; however, a transgene fragment was detected in a single sclerotium tissue recovered from a RR canola plant. This fragment was not stably integrated as the transgene fragment could not be detected in mycelia produced from the germination of this positive sclerotium. Thus, trace levels of transgene fragments may be detected, albeit at an extremely low incidence rate, from surface of sclerotia of *S. sclerotiorum* produced on the transgenic plants of canola.

Keywords: biosafety, canola, *cp4epsps*, Roundup Ready®, sclerotia, *Sclerotinia sclerotiorum*

INTRODUCTION

Canola (*Brassica napus* L.), is an agronomically important crop grown worldwide including in Europe, Asia, Canada and the United States for oil, food and animal feed. In 2007, canola acreage harvested in Canada exceeded 14.6 million acres, with over 8.7 million tonnes produced [19]. The herbicide resistant Roundup Ready® canola (RR; glyphosate tolerant, Monsanto Company, St. Louis, MO, USA) has been increasingly adopted due to decreased production costs and increased yields [13]. Roundup Ready® canola contains the transgene *5-enolpyruvylshikimate-3-phosphate synthase* from the CP4 strain of *Agrobacterium tumefaciens* (*cp4epsps*) which confers glyphosate or Roundup® resistance. Despite the overall benefits of growing transgenic crops there are numerous concerns regarding their ecological and environmental biosafety. These include evolution of herbicide resistant weeds [11, 14], outflow of herbicide resistant trait to other wild and weedy relatives [18] as well as transgene stability in various environments. The potential of movement as well as stable inheritance of transgenes from transgenic crops have previously been reported [7, 18].

Sclerotinia sclerotiorum (Lib.) de Bary is a fungal pathogen prevalent worldwide with over 400 plant hosts [2] including *Brassica napus* L. and *B. rapa* L. [20]. Past outbreaks of sclerotinia blight in North America including western Canada have resulted in severe losses to the canola industry [10, 12]. Crop rotation and chemical fungicides are methods currently used by producers in Canada for control of sclerotinia stem blight. Sclerotia of *S. sclerotiorum*, the primary source of inoculum, germinate carpogenically to release airborne ascospores for infection on canola plants [5]. Germinated ascospores often infect plants and are often killed with formation of sclerotia in the pith cavity of canola stems (Fig. 1).

Formation and development of sclerotia of *S. sclerotiorum* on canola plants provides a close association between transgene tissues of the host plant and the pathogen. Whether the sclerotia formed in diseased plants of RR canola can be a possible route for prolonged transgene survival and thereby indirectly aid in dissemination of the transgene remains unknown. The objective of the present study was to examine the presence of the *cp4epsps* transgene and transgene fragments on sclerotia of *S. sclerotiorum* collected from naturally infected stems of RR canola in the field in 2004 and 2005, using PCR and Southern hybridization techniques.

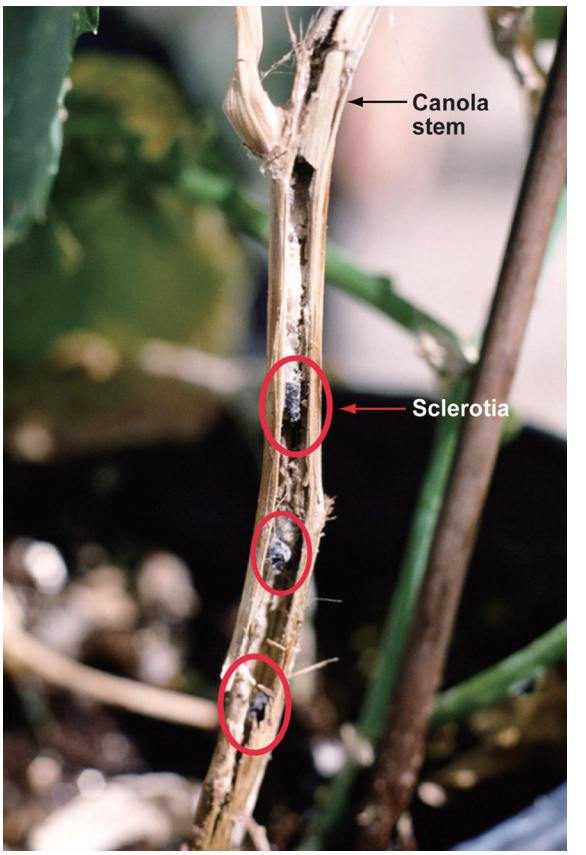

Fig. 1. Sclerotia (marked with circles) of *Sclerotinia sclerotiorum* formed in the stem of a diseased canola (*Brassica napus* L.) plant.

MATERIALS AND METHODS

Sample collection

An eight hectare field experiment was established at the Lethbridge Research Centre (Agriculture and Agri-Food Canada, Lethbridge, AB, Canada) in the year 2000 to investigate the long-term environmental impact of transgenic crops, including RR canola, approved for production in Canada. In that particular field trial, ten treatments with four replicates arranged in randomized complete block design were setup in individual plot size of 15 m × 35 m (W × L). Transgenic (RR) and non-transgenic (conventional canola; CC) canola plants were grown each year. During 2004 and 2005, stems of healthy and diseased canola plants were collected each year from each plot at the time of harvest. Sclerotia produced inside the pith cavity of

diseased stems (Fig. 1) of RR canola and CC canola were removed using a scalpel. They were washed for 10 min in distilled water, dried between two layers of sterile paper towel and rinsed again in water to remove any residual plant material from the surface. Both, the infected canola stem and the sclerotia obtained from it, were given the same identification number, stored at - 40 °C, and used for DNA extraction to test the presence of *cp4epsps* and four of the transgene fragments of *cp4epsps* in tissues of the stem and the sclerotia. A total of 48 sclerotia from RR plants and 32 from CC plants were analyzed.

In a single case where a transgene fragment was detected on one sclerotium collected from a diseased RR canola in the field, the sclerotium was surface sterilized for 60s in 70% (v/v) ethanol, air-dried, and cut in half with a sterile scalpel. Each half was placed with the cut side down on potato dextrose agar (PDA, Difco, Detroit, MI, USA) in a Petri dish. After incubation in the dark at 15 °C for 3 weeks, mycelia produced were collected aseptically and used for DNA extraction and transgene detection.

DNA extraction

Canola stem segments and sclerotia of *S. sclerotiorum* were freeze dried prior to DNA extractions. Sclerotia from individual plants were divided into three sub samples and DNA extracted independently from each sub sample.

Canola stems were weighed and ground using a ball grinder (Fritsch, Idar-Oberstein, Germany) to a fine powder (0.2 micron). Genomic DNA was extracted from diseased RR and CC stems (100 mg) using DNeasy® Plant kit (Qiagen Inc., Mississauga, ON, Canada) and eluted with 200 µl elution buffer. Genomic DNA was isolated from the leaves of 21-day old RR canola grown in phytotron facility, under standard growth conditions, using the same kit and used as a positive control for *cp4epsps* gene in PCR.

Freeze-dried sclerotia of *S. sclerotiorum* were ground to a fine powder using dry ice in a mortar and pestle. Prior to homogenization, a small portion of the original sclerotium-tissue was retained as inoculum for production of mycelia and daughter sclerotia on PDA, if required. DNA was extracted from ground sclerotia (100 mg) using the DNeasy® Plant kit (Qiagen) as per manufacturer's instructions with a modification; incubation at 65 °C for cell lysis was performed for 30 min instead of recommended 10 min and the DNA was eluted with 50 µl elution buffer. In the case of the sclerotium from RR canola with positive detection of transgene fragment, mycelia derived from this sclerotium on PDA cultures were also extracted using DNeasy® Plant kit (Qiagen).

As an additional control, DNA was extracted from sclerotia of *S. sclerotiorum* produced on diseased plants of common bean (*Phaseolus vulgaris*) grown in a field outside the trial site (near Lethbridge, Alberta) and used for PCR. DNA extractions were monitored routinely with negative controls (buffer only). The extracted genomic DNA from stems of canola and sclerotia of *S. sclerotiorum* was quantified using the Ultraspec 3000 spectrophotometer (Pharmacia Biotech Ltd., Cambridge, UK). DNA (5 µl) was resolved on a 0.7% (w/v) agarose gel prior to PCR in order to ascertain integrity of extracted DNA.

PCR analyses

PCR was carried out in triplicates and each run included a negative control containing no template DNA and an appropriate positive control (100 ng) containing RR canola leaf DNA. The DNA extracted from sclerotia produced on diseased bean plants was also used as an additional negative control. All PCR assays were conducted using the DNA Engine Dyad® thermocycler (M.J. Research Inc., Watertown, MA, USA). Each PCR reaction (50 µl final volume) contained the following (final concentrations): dNTP mix (0.2 mM), forward and reverse primer (each at 0.5 µM), $MgCl_2$ (1.5 mM), and 2.5 units of Platinum *Taq* Polymerase (Invitrogen Life Technologies, Burlington, ON, Canada) in 1 × PCR buffer.

In order to ascertain the extracted DNA from sclerotia of *S. sclerotiorum* was of PCR-quality, PCR amplification using *S. sclerotiorum*-specific primers targeting the *pg6* gene (312 bp) from the *endopolygalacturonase* gene family [8] was performed using the thermocycling conditions: 94 °C for 5 min, 30 cycles of 94 °C for 45 sec, 47.8 °C for 1 min, 72 °C for 1 min, followed by 72 °C for 7 min. The amplified fragment was confirmed by sequencing. Further, DNA from sclerotia of *S. sclerotiorum* was routinely spiked with a known amount of positive control DNA (100 pg RR leaf DNA) to rule out the presence of PCR inhibitors.

Fifty nanograms DNA template was used from canola stems and 500 ng DNA was used from sclerotia tissue. The canola stems and sclerotia of *S. sclerotiorum* were screened for the presence of *cp4epsps* gene using the forward primer

EF3 (5'-TCA CGG TGC AAG CAG CCG TCC AGC-3') and reverse primer ER_2 (5'-TCA AGC AGC CTT AGT GTC GGA GAG TTC G-3') to amplify the full-length 1363 bp *cp4epsps* (Fig. 2) using the conditions: 94 °C for 5 min, 74 °C for 5 min, 35 cycles of 94 °C for 1 min, 74 °C for 3 min, and 72 °C for 10 min.

Four smaller fragments (Fig. 2: F1, F2, F3 and F4) were amplified with the rationale being that smaller amplicons may have an increased chance of detection given that full-length genes have seldom been detected in DNA stability studies [16, 17]. The construct-specific fragments F1, F2 and F4 were amplified using one of the primers located in the promoter or terminator region whereas F3 was a gene-specific fragment. The fragments (F1, F2, F3, F4) ranged in size from 179-527 bp and amplified using the following primer sets: forward primer PF_2 (5'-AAG GCA TTC ATT CCC ATT TG-3') and reverse primer ER_1 (5'-TAA CAT CTT CAC CTT CCA AAA G-3') amplified a 527 bp fragment (F1) in the promoter/*cp4epsps*; forward primer PF_2 and reverse primer ER_3 (5'-ATT GCA GAT TCT GCT AAC TTG -3') amplified 179 bp fragment (F2) located in the promoter/CTP; forward primer EF_5 (5'-CGT GGC TGA CTT GCG TG-3') and reverse primer ER_5 (5'-CGT TAC CGA GAC CCT TAC C-3') amplified a 278 bp fragment (F3) in *cp4epsps*; forward primer EF_2 (5'-TTG ATT GCG ATG GCG ATG AAG GTG AG -3') and reverse primer TR (5'-ACA AAT GGT ACA AGA AAA ACA G-3') amplified a 420 bp fragment (F4) in the *cp4epsps*/terminator. PCR assays for all *cp4epsps* fragments were performed using the conditions: 95 °C for 5 min, 94° for 1 min, 35 cycles of 58 °C for 30 sec, 72 °C for 1 min, followed by a final extension at 72° for 10 min as described previously [16]. Of the total PCR product only 20 µl was analyzed on 1% (w/v) agarose gels containing ethidium bromide using standard procedures [15].

Care was taken during DNA extraction and PCR to avoid potential cross-contamination or DNA/amplicon carry-over. Aseptic techniques included autoclaving and treatment of equipment used for tissue grinding with 10% (v/v) bleach solution. Separate locations were used for DNA extraction, PCR and post-PCR processing.

Southern hybridization

PCR products (20 µl) were electrophoresed and transferred onto a Zeta-Probe Nylon membrane (Bio-Rad, Mississauga, ON, Canada) as described previously [9]. Following transfer, the membranes were air-dried, UV cross-linked using a UV Stratalinker 1800 (Stratagene, La Jolla, CA, USA) and processed for hybridization. Probe preparation and Southern hybridization were carried out using the Alkphos® direct labeling kit (Amersham Biosciences, Piscataway, NJ, USA). DNA for probe preparation was obtained by PCR amplification (50 µl reaction) of the fragment under investigation (F1, F2, or F4) from RR canola leaf DNA. A 5 µl aliquot of the PCR product was analyzed on a 1% (w/v) agarose gel, and the remaining product purified using the QIAquick® PCR Purification kit (Qiagen). The probe was labeled, membranes hybridized and exposed according to manufacturer's instruction and as standardized previously [17]. The membranes were exposed typically for 2 h but the exposure time was adjusted depending upon the signal strength.

RESULTS AND DISCUSSION

DNA extracted from canola stems was of consistently good quality and high molecular weight (Fig. 3A). Initially,

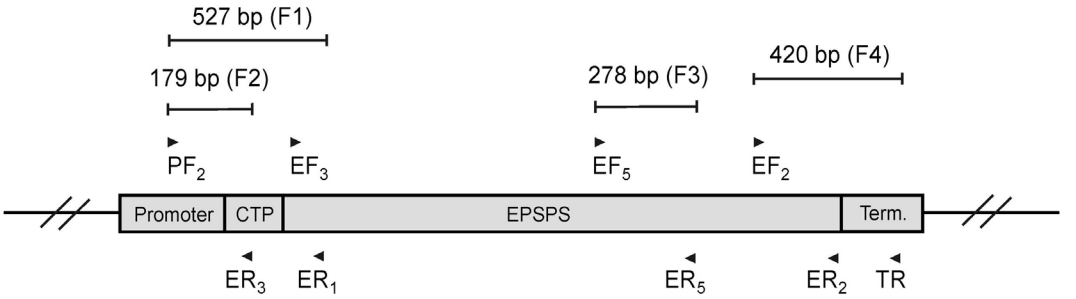

Fig. 2. Schematic representation of the *cp4 5-enolpyruvylshikimate-3-phosphate synthase* (*cp4epsps*) construct showing locations of the designed primers and fragments amplified (F1-F4). Promoter, chloroplast transit peptide (CTP), *cp4epsps* and terminator (Term.) regions are shown (drawing not to scale).

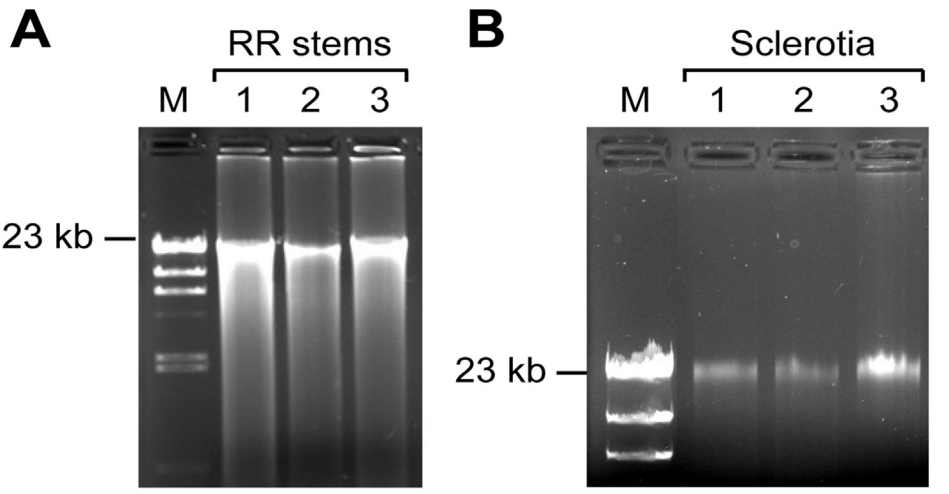

Fig. 3. Gel electrophoresis of genomic DNA isolated from A. diseased Roundup Ready® canola stems (0.7% agarose gels, 5 µl DNA extracted using 100 mg tissue), Lanes 1-3 represent extractions from three different stems. B. sclerotium from diseased Roundup Ready® canola stems (0.7% agarose gels, 10 µl DNA extracted using 100 mg tissue), Lanes 1-3 represent extractions from three different tissues. For panels A-B, Lane M: lambda DNA/*Hind* III marker.

when same tissue weight (100 mg) was used, total DNA yields were lower from sclerotia of *S. sclerotiorum* as compared to canola stems. This was due to inadequate lysis because of the hard exterior of sclerotia; thereupon the procedure was modified to include freeze-drying, grinding using dry ice and increased time for cell lysis. Using these modifications, we obtained better homogenization and tissue disruption allowing for higher DNA yields.

PCR analyses

PCR analysis of CC and RR canola stems for the 1363 bp *cp4epsps* confirmed the presence of transgene in stems of diseased RR canola plants from which the sclerotia of *S. sclerotiorum* were recovered (Fig. 4A). As expected, all CC plants showed absence of the transgene. The *pg6* was amplifiable from all sclerotial samples indicating the absence of PCR inhibitors (Fig. 4B) and was confirmed by sequencing. Further, the sclerotia recovered from diseased bean plants tested negative for complete transgene and fragments.

PCR for the *cp4epsps* did not show the presence of the full-length transgene in any of the sclerotia formed in infected RR canola stems collected from the field in both years of 2004 and 2005 (Fig. 5A). The absence of the complete 1363 bp *cp4epsps* transgene has been previously shown in *in vitro* cultures where complete transgene was found to be rapidly degraded upon release [1, 16, 17].

Despite a low limit of detection by PCR (50-60 pg)

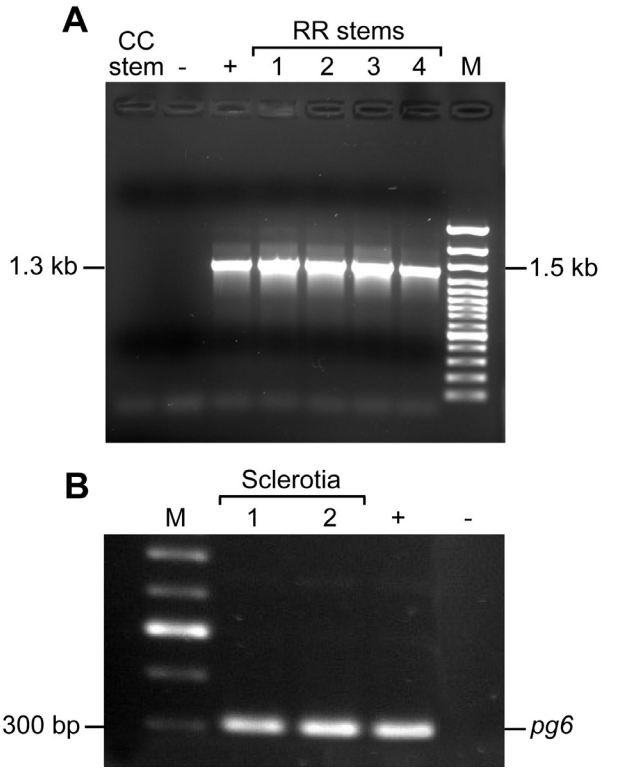

Fig.4. A. PCR analysis of 1363 bp *cp4epsps* from diseased Roundup Ready® canola stems, Lane CC: conventional canola (negative extraction control), Lanes 1-4: PCR product from four diseased stems. Lane +: positive PCR control (Roundup Ready® leaf DNA as template) B. PCR amplification of the *pg6* gene (312 bp) of the *endopolygalacturonase* gene family, Lanes 1 and 2: two different sclerotia samples, Lane +: positive PCR control (DNA from sclerotia of conventional canola stem as template); for panels A-B, Lane M: 100 bp DNA ladder, Lane -: negative PCR control (no DNA template).

and Southern hybridization (< 5 pg), fragments F1, F3 and F4 were not detected from sclerotial samples analyzed from both years (Fig. 5B-D). However, fragment F2 was detected from a single sclerotium collected from RR stem in 2005 by PCR (Fig. 6A) and later confirmed by Southern hybridization (Fig. 6B) and sequencing of the fragment. The PCR was repeated several times on this positive sample using the original genomic DNA and was still found to test positive for the F2 fragment. We found that additional sclerotia tissues from the same plant did not show the presence of fragment F2 (Fig. 6B). In order to interpret the observed transgene presence from this single sclerotium, a sub sample of the original sclerotium tissue was inoculated on PDA and the mycelia from the 3-week-old PDA culture was tested for the presence of F2 and other fragments. Results showed that the mycelia derived from this field collected sclerotium were negative for fragment F2 upon PCR and Southern hybridization, suggesting lack of stable integration of F2 in the tissue of this sclerotium. Therefore, detection of F2 fragment from the original sclerotium collected from a diseased plant of RR canola suggests that trace levels of the transgene fragment may adhere to the sclerotium surface and can be detected at very low frequencies from this tissue. From the F2 positive sclerotia DNA, we did not detect the presence of the other fragments (F1, F3 or F4).

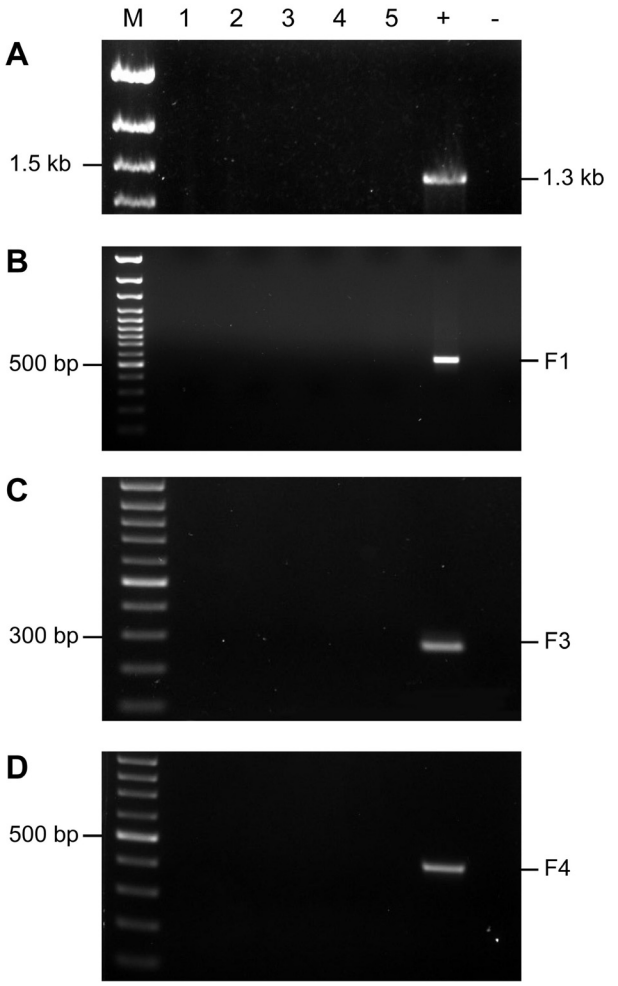

Fig. 5. Representative gel for PCR amplification of *cp4epsps* (1363 bp) and three transgene fragments F1, F3, F4 in sclerotia collected from diseased Roundup Ready® stems. A: absence of the 1363 bp *cp4epsps*. Lanes 1, 2, 4, and 5: PCR products from four different sclerotia. Lane 3: blank (no PCR product loaded). B: absence of fragment F1 from sclerotia samples. Lanes 1-5: PCR products from five different sclerotia samples. C: absence of fragment F3 from sclerotia. Lanes 1- 4: PCR products from four different sclerotia samples. Lane 5: blank. D: absence of fragment F4 from sclerotia. Lanes 1-5: PCR products from five different sclerotia samples. For panels A-D, Lane M: 100 bp DNA ladder, Lane +: positive control (Roundup Ready® leaf DNA as template), Lane - : negative control (no DNA template).

This study was aimed to characterized trace levels of transgenic DNA from sclerotia of *S. sclerotiorum* collected over two years. It is of significance to understand the transgene stability aspect upon over wintering of sclerotia of *S. sclerotiorum* in the soil, as it is among the concerns related to long term impact of seeding transgenic crops.

The absence of the complete transgene in sclerotia from RR stems can be attributed to several plant defense mechanisms. It is known that plants commonly respond to fungal infections by hypersensitive response and release of reactive oxygen species to stall the pathogen infection [6]. Characteristics of plant pathogen response include nuclear DNA fragmentation which has been documented to occur within 24 hours of infection in some plants [21]. Research suggests that the hypersensitive responses and digestive secretions degrade transgenic DNA before plant pathogens could have a chance of uptake. *S. sclerotiorum* secretes oxalic acid during infection and is a key pathogenicity factor of the fungus [3] since it acidifies plant tissues surrounding the site of infection and causes tissue injury [4]. The associated lower pH at the time of infection is ideal for the functioning of cellulolytic and pectinolytic enzymes that assist in the continued breakdown of the host plant tissues [22], and may also contribute to the degradation of cellular components, including transgene DNA, thereby preventing the outflow of transgenes among species. The plant survival mechanisms limit the uptake of transgenic DNA into *S. sclerotiorum*.

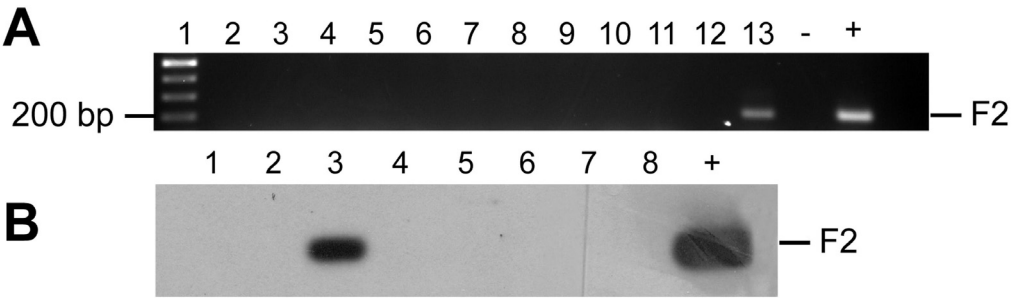

Fig. 6. A: PCR amplification of fragment F2 from sclerotia tissue. Lanes 2-9 and 11: sclerotia collected from RR stems; Lane 10: sclerotia from diseased conventional canola stem (negative control); Lane 12: blank (no PCR product); Lane 13: single sample testing positive for F2; Lane 1: 100 bp DNA ladder. Lane -: negative control (PCR using DNA from sclerotia of *Phaseolus vulgaris*) and Lane +: positive control (Roundup Ready® leaf DNA as template).
B: Southern hybridization for detection of F2 from single positive sample. Lane 1: additional DNA extraction from sclerotia tissue recovered from the same plant; Lane 3: original sample testing positive on PCR; Lane 5: DNA extracted from mycelia; Lane 7: negative control Lane - : negative control (PCR using DNA from sclerotia of *Phaseolus vulgaris*); Lanes 2, 4, 6, 8 are empty lanes and have no PCR products.

This study reveals that the trace level of *cp4epsps* transgene detected in one sclerotium of *S. sclerotiorum* formed in the stem of a Roundup Ready® canola plant is possibly due to adhesion of the transgene on the surface of this sclerotium and that traces of transgenic DNA may persist upon long term host-pathogen association, albeit at extremely low incidence rates.

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摘 要

Sharma, R.、黃鴻章、John, S. J.、Munns, K.、Blackshaw, R. E. 2010. 由罹患菌核病之抗嘉磷塞除草劑基因改造油菜上的菌核可偵測出微量的 *cp4epsps* 轉殖基因核酸. 植病會刊 19：1-8.

自 1990 年代初期引進基因改造作物以來，基因改造作物的種植面積已呈穩定成長。然而，關於基因改造作物之轉殖基因的持久性與穩定性，以及其後續外流到環境中之疑慮則一直受到關注。Roundup Ready® (RR) 的基因改造油菜 (*Brassica napus* event RT73) 乃是帶有 *cp4* EPSP 合成酶 (*cp4epsps*) 基因以具有抗嘉磷塞 (glyphosate) 除草劑的特性。菌核病菌 (*Sclerotinia sclerotiorum*) 是造成油菜莖枯與莢腐的重要病原菌。本研究的目的在於檢測田間罹患菌核病的 RR 基因改造油菜，其莖部所形成的菌核是否帶有 *cp4epsps* 轉殖基因的核酸。在二年的研究期間，於收穫時由自然感染菌核病的 RR 基因改造油菜與非基改油菜莖上所收集的菌核，利用聚合酶連鎖反應〔PCR，檢測極限爲 50 皮克 (微微克，pg)〕與南方氏雜合反應 (檢測極限小於 5 皮克) 來篩選是否帶有 1363 鹼基對長度的 *cp4epsps* 或更短轉殖基因片段。結果顯示在所有分析的菌核中皆未檢測出完整的 *cp4epsps* 轉殖基因；然而，在一株 RR 基因改造油菜植株所收集之單一菌核組織中可測到轉殖基因的部份片段。此基因片段並未穩定嵌入菌核中，因爲由該正反應的菌核所發芽而長成之菌絲中，並無法檢測到轉殖基因的片段。因此，由罹患菌核病之基因改造油菜上的菌核表面雖可測得微量的轉殖基因片段，但其檢出率極低。

關鍵詞：生物安全、油菜、*cp4* EPSP 合成酶基因、抗嘉磷塞除草劑 (Roundup Ready®)、菌核、菌核菌 (*Sclerotinia sclerotiorum*)