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SHORT COMMUNICATION

Stable *Bacillus thuringiensis* (Bt) toxin content in interspecific F_1 and backcross populations of wild *Brassica rapa* after Bt gene transfer

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Abstract

Stable expression of a transgene may lead to increased fitness for wild plants after acquiring the transgene via crop–weed hybridization. Here, we investigate the stability of Bt toxin content in wild *Brassica rapa* acquiring the Bt gene from Bt *Brassica napus*. The Bt toxin content in nine Bt-expressing *B. napus* lines was 0.80–1.70 μg/g leaf tissue throughout the growing season. These nine lines were crossed with three accessions of wild *B. rapa* and the Bt gene was successfully transferred to interspecific hybrids (F_1) and successive backcross generations (BC_1 to BC_4). The Bt toxin level in F_1 and BC progenies containing the Bt gene remained at 0.90–3.10 μg/g leaf tissue. This study indicates that the Bt gene can persist and be stably expressed in wild *B. rapa*.

Keywords: *Bacillus thuringiensis* (Bt) toxin, backcross, *Brassica napus*, interspecific hybridization, transgene transfer, wild *B. rapa*

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Introduction

With the commercial release of transgenic crops, transgene movement to wild relatives via crop–weed hybridization has raised concerns about potential ecological risks (Mikkelsen *et al*. 1996; Chèvre *et al*. 1997; Snow 2002). One of the model systems for risk assessment is transgenic *Brassica* crops and their wild relatives, because *Brassica* crops have various closely related species present in cultivated fields, some of which are economically important weeds (Jørgensen *et al*. 1996a; Holm *et al*. 1997; Snow *et al*. 1999). The ecological risks associated with crop–weed hybridization in this model system have been extensively studied, results showed that interspecific hybrids between wild *Brassica rapa* and *B. napus* crops were successfully produced, and hybrids could further backcross with wild *B. rapa* (Jørgensen & Andersen 1994; Mikkelsen *et al*. 1996; Snow *et al*. 1999), even under field conditions (Jørgensen *et al*. 1996a). In most cases, research has focused mainly on initial interspecific hybridization. Experimental data concerning transgene behaviour in wild relatives including introgression and stability of the transgene in successive backcross generations are lacking (Linder & Schmitt 1994; Jørgensen *et al*. 1996b; Mikkelsen *et al*. 1996). This is especially important with fitness-enhancing transgene(s) in wild plants, because stable expression of a transgene conferring increased fitness may have a substantial ecological impact (Kareiva *et al*. 1994; Linder & Schmitt 1994; Stewart *et al*. 1997; Burke & Rieseberg 2003). As an example, the *Bacillus thuringiensis* (Bt) gene, which can be used to develop Bt crops for insect control, may confer a fitness advantage in the wild plant species acquiring the Bt gene via crop–weed hybridization. Stewart *et al*. (1997) provided preliminary evidence showing that insect resistance conferred a fitness advantage to Bt *B. napus* plants, when Bt *B. napus* and nontransgenic plants were subjected to insect pressures. Therefore, it is conceivable that if wild plants acquire a Bt gene and stably express Bt toxin, they may become more invasive.

Although no Bt *Brassica* crops have been commercially released, a number of transgenic Bt *Brassica* lines have been

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developed and their toxicity to target insect pests confirmed (Metz *et al*. 1995; Stewart *et al*. 1996; Ramachandran *et al*. 1998). However, when insect pests and Bt crops come into contact during the growing season, constant exposure of insect pests to Bt toxin expressed in Bt crop plants may facilitate target insect pests developing resistance (Tabashnik 1994; Hokkanen & Wearing 1995; Tabashnik *et al*. 1997b). Therefore, knowledge of the stability of Bt toxin content in Bt *Brassica* crop plants throughout the growing season is needed. Furthermore, interspecific hybridization between *B. napus* crops and their wild relatives, especially wild *B. rapa*, readily occurs, and concern regarding the fitness effects of the Bt gene in wild plants has been raised (Stewart *et al*. 1997). Thus, as an initial step for assessing Bt gene fitness effects in wild plants, monitoring *Bt* gene persistence and expression in interspecific hybrids and backcross progenies after wild *B. rapa* plants acquire the Bt gene from Bt *Brassica* crops is required. In this study, we investigated Bt toxin content in nine transgenic Bt-expressing *B. napus* lines throughout a plant growing season and crossed these nine lines with three wild *B. rapa* accessions to monitor the persistence and stability of the *Bt* gene in F_1 hybrids and backcross progenies of wild *B. rapa*.

Materials and methods

Plant materials

Harper *et al*. (1999) described the development of transgenic green fluorescent protein (GFP)-Bt *Brassica napus* lines through transforming Westar (a *B. napus* cultivar) with a transgene construct (*mGFP5er–Bt cry1Ac*), designed to use the GFP gene to monitor the Bt (*Bacillus thuringiensis*) gene. Thus, the screening of putative hybrids for the Bt gene was facilitated by GFP visualization. Nine homozygous Bt-expressing *B. napus* lines (named GT1–9) were developed by selfing nine independent transformation events, each containing the Bt gene at a single locus (Harper *et al*. 1999). These GT *B. napus* lines were used to test the stability of Bt toxin content during a growing season in a field trial and in initial interspecific hybridization with wild *B. rapa*. Two wild *B. rapa* accessions, 2975 and 2974, from Waterville (45°16′ N 71°54′ W) and Milby (45°19′ N 71°49′ W), Quebec, Canada, respectively, and one accession, named CA, from Irvine (33°40′ N 117°49′ W), California, USA (courtesy of Art Weiss), were used in interspecific hybridization and successive backcross generation development.

Development of interspecific F_1 and successive backcross populations

Each of the three wild *B. rapa* accessions (used as pollen recipients), 2975, 2974 and CA, was manually crossed with each of the nine GT *B. napus* lines to develop F_1 plants. Putative hybrids (2–3 weeks old) were screened for GFP using a hand-held UV lamp (UVP model-B-100AP, 100 W : 365 nm) as described by Halfhill *et al*. (2001). After screening for the Bt gene by visualizing GFP expression, randomly selected F_1 hybrids were used as pollen donors and backcrossed with corresponding wild *B. rapa* accessions, 2975, 2974 and CA, respectively, to develop the BC_1 generation. Subsequently, the same strategy was utilized to develop BC_2, BC_3 and BC_4 populations. F_1 and BC_1–BC_4 progenies derived from each of the nine GT *B. napus* lines were used to monitor the persistence and expression of Bt gene in wild plants. The chi-square test was used to determine whether the Bt gene distribution in BC populations followed a Mendelian model. All F_1 and BC plants were grown in 5-inch pots filled with standard potting soil and maintained in a growth chamber at 22 / 16 °C (day/night) with 16-h daylight under cool-white fluorescent lights. From F_1 hybrids and BC_1–BC_4 progenies containing the Bt gene derived from each of the GT *B. napus* lines, 3–6 plants were randomly selected from each generation and Bt toxin analysis was conducted for the individual plants at the growth stage of 3–5 true leaf. Leaves (third from top) were used for Bt toxin quantification and original plants of wild *B. rapa* accessions were used as control.

Field experiment

Field testing of Bt toxin level in the nine GT *B. napus* lines was carried out at the Central Experimental Farm of the Eastern Cereal and Oilseed Research Centre (45°25′ N 75°43′ W), Agriculture & Agri-Food Canada, Ottawa in 2001. The nine GT *B. napus* lines and Westar (control) were randomly assigned to 6-m long rows that were spaced 22-cm apart. Seeds were sown on 21 May 2001; plant density was approximately 180 plants per row. Six plants were randomly selected from each of the nine GT *B. napus* lines and Bt toxin analysis was conducted for the individual plants at three different growth stages [3–5 true leaf (June 15, 2001), flowering time (July 10, 2001) and pod formed (July 27, 2001)]. Leaves (third from top) were collected for Bt toxin quantification and Westar was used as control. To comply with regulations for confined field trials of novel plant traits of the Canadian Food Inspection Agency (CFIA), a 10-m wide buffer zone with Westar was set up to trap transgenic pollen, and all plants tested in the experiment were cut before seeds matured to avoid spreading transgenic seeds.

Bt toxin analysis

Leaf samples (two punches of leaf disks, clipped using a 1.5-mL microtube lid) from individual plants were weighed and ground, then Bt toxin was extracted and analysed

following the manufacturer's instructions (EnviroLogix Cry1Ab/Cry1Ac Plate Kit). Bt toxin content (μg per gram of leaf tissue) was expressed as the means ± the standard errors of the means.

Results and Discussion

Bt toxin content in nine transgenic GT Brassica napus *lines*

When the nine transgenic GT *Brassica napus* lines were grown in the field trial, the average Bt toxin content at the growth stage of 3–5 true leaves was 0.8–1.6 μg / g leaf tissue, with GT9 at 1.6 μg / g and GT1 at 0.8 μg / g (Fig. 1A). We also monitored the dynamics of Bt toxin content in all nine GT *B. napus* lines throughout the plant growing season, the results showed that the level of Bt toxin was close to or greater than 1.0 μg / g of leaf tissue in all GT *B. napus* lines. As plant growth progressed, the level of Bt toxin content increased slightly (Fig. 1B). In an insect bioassay study using the same GT *B. napus* lines, Halfhill *et al*. (2001) observed that Bt toxin expressed in these nine GT *B. napus* lines was highly insecticidal to corn earworm (*Helicoverpa zea*) and effectively prevented herbivory damage. Stewart *et al*. (1996) reported similar results showing a high mortality of diamondback moth (*Plutella xylostella*) and cabbage looper (*Trichoplusia ni*), when transgenic Bt *B. napus* was fed to the insects. These results suggest that Bt toxin expressed in transgenic Bt *B. napus* can effectively control target insect pests, such as diamondback moth, a pest of global significance in a variety of crop systems including *Brassica* crops. Stable Bt toxin content in the nine GT *B. napus* lines shown in this study implies that target insect pests could be controlled throughout the growing season. However, this may raise a concern that constant exposure to Bt toxin expressed in Bt crop plants would lead to faster development of resistance in insect pests, because several Lepidopteran species have been reported developing resistance to Bt toxin in both field and laboratory tests (Lambert & Peferoen 1992; Tabashnik 1994; Huang *et al*. 1999), and even one autosomal recessive gene conferred high resistance to four Bt toxins (*Cry1Aa*, *Cry1Ab*, *Cry1Ac* and *Cry1F*) in a strain of the diamondback moth (Tabashnik *et al*. 1997a).

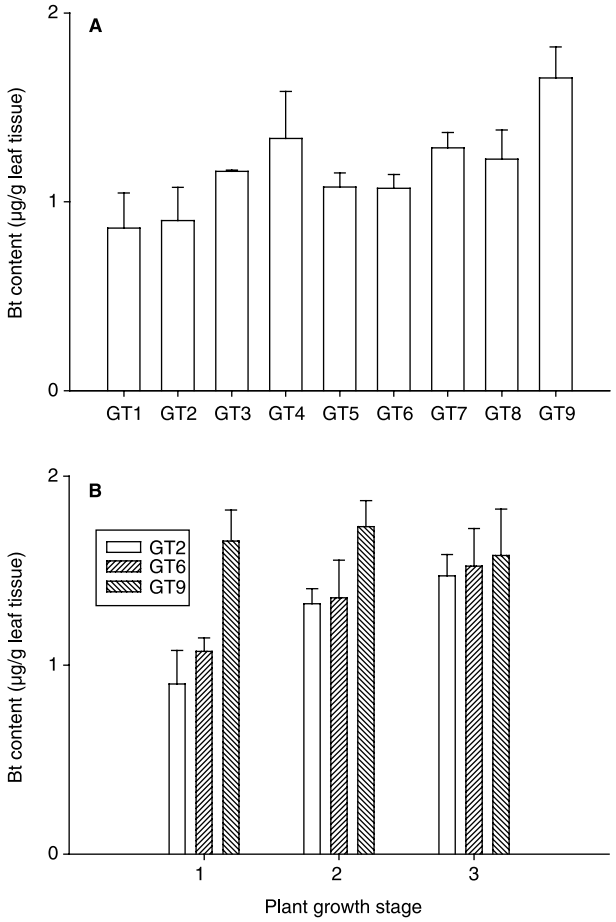

Fig. 1 Means (SE) of Bt toxin content in nine GT *Brassica napus* lines at the stage of 3–5 true leaf in the field trial (A) and means (SE) of Bt toxin content in three GT *B. napus* lines (GT2, GT6 and GT9) during the plant growing season (B). Stage 1: 3–5 true leaf; Stage 2: flowering time; and Stage 3: pods were formed.

Bt content in interspecific F_1 and successive backcross generations

We investigated the persistence and stability of the Bt gene in wild *B. rapa* by crossing the nine GT *B. napus* lines with three wild *B. rapa* accessions, 2975, 2974 and CA. Interspecific hybrids (F_1) were successfully produced and backcross progenies (BC_1–BC_4) containing the Bt gene were also developed in all three accessions, suggesting that the Bt gene can persist in wild *B. rapa* for generations after initial transfer. The Bt toxin level in F_1 hybrids and successive backcross progenies containing the Bt gene was close to or greater than 1.0 μg / g of leaf tissue in all generations, similar to or higher than that of the same GT *B. napus* lines from which the F_1 and backcross progenies acquired the Bt gene (Fig. 2). Some variation of Bt toxin content in F_1 and backcross generations containing the Bt gene derived from the same GT *B. napus* lines was observed. This may be caused by differences in plant growing conditions, which has been reported as a factor contributing to variation in the Bt toxin level in other transgenic Bt *B. napus* lines (Ramachandran *et al*. 1998).

Bt toxin detection in the F_1 and backcross progenies of wild *B. rapa* indicates stable Bt gene expression in the wild plants. It is worthwhile noting that by investigating inheritance of the Bt gene in backcross generations, we observed that the Bt gene from some GT *B. napus* lines such as GT2

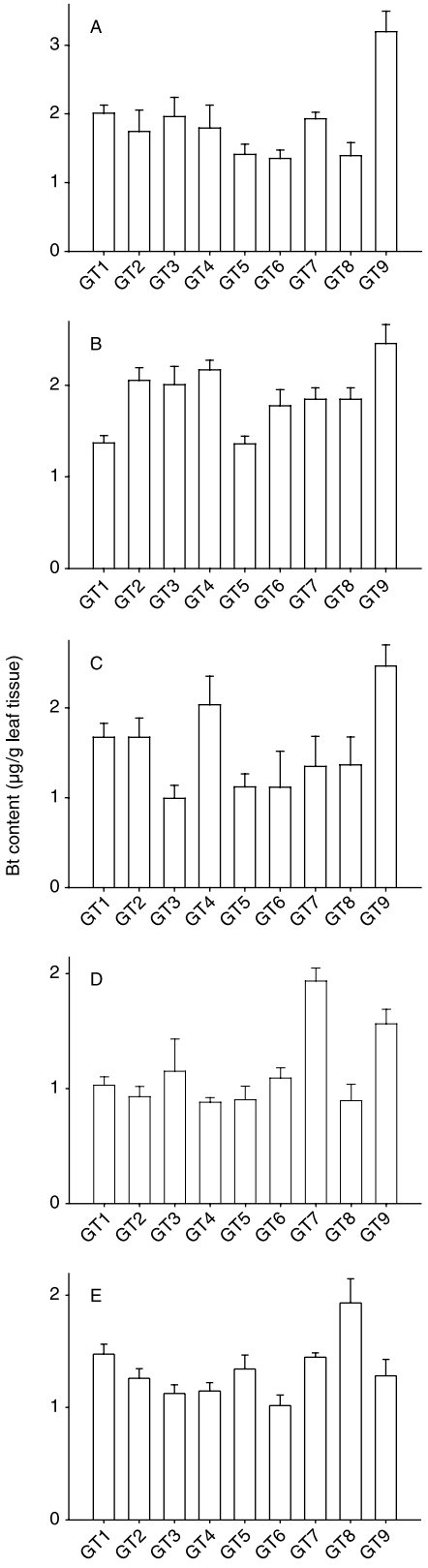

Fig. 2 Means (SE) of Bt toxin content in F_1 (A), BC_1 (B), BC_2 (C), BC_3 (D) and BC_4 (E) progenies derived from the cross of the accession of 2975 × nine GT *Brassica napus* lines, respectively.

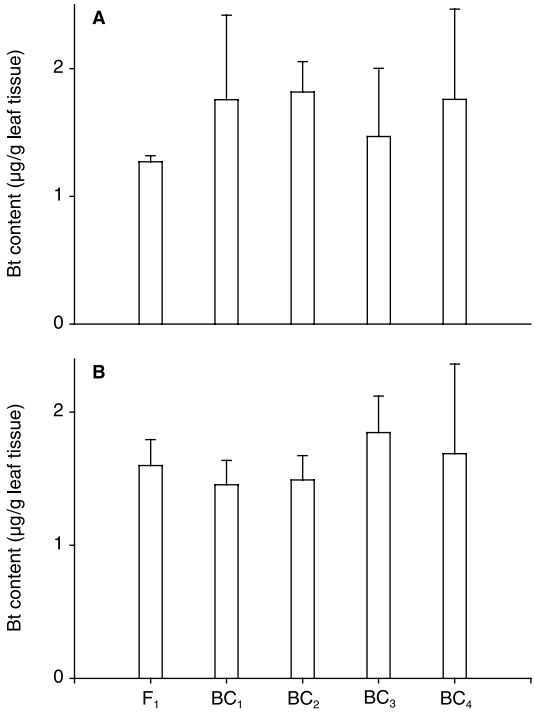

Fig. 3 Means (SE) of Bt toxin content in F_1 and successive backcross (BC_1–BC_4) progenies derived from the cross of the accession of 2974 × GT2 (A) and the accession of CA × GT7 (B).

followed a Mendelian genetic pattern in BC_2–BC_4 generations of all three *B. rapa* accessions. Of more than 100 progenies produced in each generation, almost 50% had the Bt gene (chi-square test, $P > 0.05$), indicating integration of the Bt gene into the wild *B. rapa* genome (data not shown). However, the Bt gene from other GT *B. napus* lines such as GT7 did not follow a Mendelian genetic model, < 20% of the progenies produced in each generation had the Bt gene (chi-square test, $P < 0.001$). This suggested that the Bt gene was still located on an additional chromosome in the backcross generations of all three *B. rapa* accessions (data not shown). Nonetheless, stable Bt toxin content was detected in F_1 hybrids and backcross progenies (BC_1–BC_4) derived from these two GT *B. napus* lines in 2975 accession (Fig. 2). This was also true when Bt toxin content was measured in the F_1 and backcross generations (BC_1–BC_4) derived from these two GT *B. napus* lines in 2974 and CA accessions (Fig. 3A and 3B). These results demonstrated that all F_1 and backcross progenies containing the Bt gene showed a stable Bt toxin content, no matter whether the Bt gene was integrated into the genome of wild *B. rapa* or located on an additional chromosome.

Our results showed the persistence and stable expression of Bt gene in F_1 hybrids and successive backcross progenies of wild *B. rapa* after initial Bt gene transfer from the GT *B. napus* lines. Other wild relatives of *B. napus* crops, such as *Raphanus raphanistrum*, have also been reported

acquiring transgene(s) through interspecific hybridization with transgenic *B. napus* in field trials (Chèvre *et al*. 2000). Therefore, there is a need to investigate the stability of Bt gene in those wild relatives that can cross with *Brassica* crops. Furthermore, one of the ecological concerns regarding transgene flow is the potential of wild plants for increased fitness after the acquisition of a transgene (Burke & Rieseberg 2003). As a model system for studying ecological risks associated with transgene flow via crop–weed hybridization, Bt gene flow from Bt *Brassica* crops to their wild related species and the ecological implications of stable Bt toxin expression in wild plants need to be addressed further.

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