Segregation and Efficacy of the *cry1Ia1* Gene for Control of Potato Tuberworm in Four Populations of Cultivated Potato

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ABSTRACT The potato (*Solanum tuberosum* L.) cultivar 'SpuntaG2' contains a single copy of the *Bacillus thuringiensis* (*Bt*) *cry1Ia1* gene and controls potato tuberworm (*Phthorimaea operculella* Zeller, Lepidoptera: Gelechiidae). Two potato cultivars and two breeding lines were crossed with SpuntaG2 creating four populations used to study *cry1Ia1* segregation and efficacy. The *cry1Ia1* gene segregated in each of the four populations with a 1:1 ratio. All progeny that were polymerase chain reaction positive for the *cry1Ia1* gene had no surviving larvae and no leaf mining in detached leaf assays after 72 h. These results support previous evidence that SpuntaG2 carries a single copy of the *cry1Ia1* gene and that transmission of the transgene from parent to progeny is not restricted and follows expected Mendelian segregation ratios. Based on detached leaf assays, the efficacy of the *cry1Ia1* gene is retained through sexual transmission. If the SpuntaG2 *cry1Ia1* insertion event is deregulated for commercial use, SpuntaG2 could be used for conventional breeding and the progeny carrying the SpuntaG2 event would also be available for commercial use.

KEY WORDS *Bacillus thuringiensis*, *Bt*, *Solanum tuberosum*, *Phthorimaea operculella*, transgenic

Potato tuberworm (*Phthorimaea operculella* Zeller, Lepidoptera: Gelechiidae) has long been recognized as a serious pest of potato (*Solanum tuberosum* L.) in tropical and subtropical areas of the world. However, it has recently emerged as a significant pest of potatoes in the Pacific Northwest of the United States, an area that produces >55% of the nations' potatoes (Rondon et al. 2007, Guenthner 2010). Potato tuberworm can damage potato plants in the field by mining the leaves and stems, and it can also mine tubers in storage. Significant mining of leaves and stems can result in yield losses but the most significant losses occur because of tuber infestation. Tuberworm damage of potato tubers can render them unmarketable as a fresh-market product and can lead to increased susceptibility to bacterial and fungal infection during storage and transit (Visser 2004, Rondon 2010). Furthermore, because potato tuberworm larvae are considered a foreign substance there is a zero-tolerance policy for their presence in raw processing potato products (Rondon et al. 2007).

There are no commercial potato cultivars that are resistant to potato tuberworm; thus, control of this insect relies on the use of a variety of broad spectrum insecticides. Alternative means of potato tuberworm control are desirable for several reasons including: the cost of chemical control, the potential for the development of insect resistance to insecticides, and the potential negative effects of insecticides on food supplies and the environment. Vigilant scouting and cultural practices such as harvesting soon after vine killing, maintaining soil moisture after vine killing, hilling after vine-kill, and limiting tuber exposure time in the field have been recommended for potato tuberworm control (Shelton and Wyman 1979, Clough et al. 2008, Rondon et al. 2009).

Biopesticides derived from toxins present in the naturally occurring, soil bacterium *Bacillus thuringiensis* (*Bt*) are effective against potato tuberworm (Hamilton and Mcdonald 1990) but are limited in their effectiveness because of a short residual activity. To overcome this problem, and the high production cost of *Bt* insecticides, transgenic plants were engineered to constitutively express *Bt* genes (Vaeck et al. 1987). One such gene is the *cry1Ia1* gene (previously referred to as the *cryV* gene) that is effective against lepidopteran insects (Douches et al. 1998, Westedt et al. 1998).

The potato cultivar 'SpuntaG2' was developed to demonstrate the efficacy of the *cry1Ia1* gene to combat potato tuberworm in South Africa. Subsistence-

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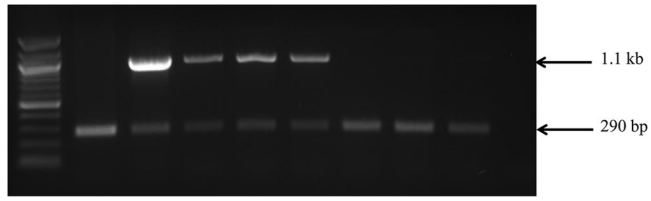

Fig. 1. Agarose gel separation of *cry1Ia1* PCR products from SpuntaG2, MSL211–3, and MSX973 progeny. Lane 1: 100 bp DNA standard, Lane 2: MSL211–3, Lane 3: SpuntaG2, Lanes 4–9: MSX973 progeny segregating for the *cry1Ia1* transgene, Lane 10: water control. The *cry1Ia1* band is 1.1 kb and the internal control band (*BCH*) is 290 bp.

level potato growers in South Africa are either unable to afford or have no access to insecticides and often store their potatoes in huts or shacks that provide easy access for potato tuberworm moths. Furthermore, because there are no chemicals registered for control of potato tuberworm postharvest, growers can suffer severe postharvest losses (Nel et al. 2001). Extensive molecular, safety, and efficacy studies have been conducted in an effort to deregulate SpuntaG2 in South Africa (Douches et al. 2010a, Quemada et al. 2010, Zarka et al. 2010).

SpuntaG2 contains a single copy of the *cry1Ia1* gene and has complete resistance to potato tuberworm (Rondon et al. 2009, Douches et al. 2010b, Zarka et al. 2010). If SpuntaG2 is deregulated, it can be used as a source of potato tuberworm resistance for variety development without further regulatory analysis required for the resultant progeny. Furthermore, the strong correlation between the presence of the *cry1Ia1* gene and potato tuberworm mortality could enable marker-assisted breeding for potato tuberworm resistance. To develop potato cultivars carrying the *cry1Ia1* gene for potato tuberworm resistance and to study the inheritance and efficacy of the *cry1Ia1* gene in progeny, SpuntaG2 was crossed to two commercial potato cultivars and two potato breeding lines from the Michigan State University potato breeding program. Four populations segregating for the presence of the *cry1Ia1* gene were created. We report here on the segregation ratios of the *cry1Ia1* gene in each of the four populations and the efficacy of the individual progeny against potato tuberworm in detached leaf assays.

Materials and Methods

Plant Material. Four populations segregating for the *cry1Ia1* gene were developed from the following crosses: SpuntaG2 × 'MSL211–3′ (MSX970), SpuntaG2 × 'MSN105–1′ (MSX971), SpuntaG2 × 'Silverton Russet' (MSX972), and SpuntaG2 × 'Missaukee' (MSX973). MSL211–3 and MSN105–1 are both high-yielding, bright-skinned, round, white potatoes that fit both the South African market and the United States tablestock market. Missaukee and Silverton Russet were selected to deploy the *cry1Ia1* gene in a potato chip processing and russet cultivar, respectively. Population sizes were 74, 97, 22, and 30 for MSX970, MSX971, MSX972, and MSX973, respectively. Progeny minitubers were planted in potting mix (SureMix, MI Grower Products Inc., Galesburg, MI) and grown in a greenhouse to be used for DNA extraction and detached leaf tests. The temperature in the greenhouse ranged from 18 to 25°C and a 16-h photoperiod (30 μmol/m^2/s) was maintained using high-pressure sodium lights.

DNA Extraction and Analysis. There were 100 mg of leaf tissue collected for DNA extraction using DNeasy Plant Mini Kits (Qiagen, Valencia, CA). Polymerase chain reaction (PCR) was conducted on each line to identify the presence/absence of the *cry1Ia1* gene. Each reaction consisted of 12.5 μl GoTaq Green Master Mix 2X (Promega, Madison, WI), 1 μl (5 mM) of each *cry1Ia1* primer (*cry1Ia1-F:* 5′-GTGGGTCACGAACTTCCAGT-3′; *cry1Ia1-R:* 5′-CCTAAGGGCAAGAACCAATG-3′), 0.3 μl (1.5 mM) of each BCH primer (BCH-F: 5′-CATGACATAGTTTGAATTTTGAGTC-3′; BCH-R: 5′-CGTTTGGCGCTGCCGTAAGTT-3′), and 7.9 μl nuclease free water for a total reaction volume of 25 μl. BCH primers amplify the beta-carotene hydroxylase gene in potato (290 bp band) and are used as an internal control to verify the quality of the results. The PCR cycling parameters were as follows: 5 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 50°C, and 90 s at 72°C and a 10 min final extension at 72°C. PCR products were separated and visualized on a 1% agarose gel with ethidium bromide staining. Each progeny was scored for the presence/absence of the *cry1Ia1* band (1.1 kb). The segregation ratio of each population was analyzed via Pearson χ^2 Goodness of Fit test (JMP 9.0, SAS Institute Inc., Cary, NC) to determine fit to the expected 1:1 segregation ratio.

Detached Leaf Bioassays. When plants were at a six-leaf stage, leaves were removed and the petioles of the excised leaves were placed in water-filled vials and sealed with parafilm to maintain leaf turgor. The detached leaves were placed on filter paper in 20 × 150 mm petri dishes and five, first stage larvae from a colony maintained at Michigan State University (Cooper et al. 2009) were placed near the leaf midrib using a brush. The petri dishes were covered and held at 25 ± 2°C with a light intensity of 25 μmol/m/s. Each dish represented one replication and each line was replicated three times. For each assay, leaf mining was evaluated (yes or no) and the number of surviving larvae was recorded after 72 h. Leaf mining was defined as more than one larval body length.

Table 1. *cry1Ia1* segregation results for four populations derived from 'SpuntaG2'

Family	Pedigree		Number of progeny[a]			χ^2 *P* value[b]
	Female	Male	*cry1Ia1* +	*cry1Ia1* −	Total	
X970	SpuntaG2	MSL211–3	37	37	74	1
X971	SpuntaG2	MSN105–1	35	51	86	0.0845
X972	SpuntaG2	Silverton Russet	13	8	21	0.2752
X973	SpuntaG2	Missaukee	14	12	26	0.6949
Overall			99	108	207	0.5316

[a] Presence/absence of the *cry1Ia1* gene determined by PCR.
[b] Pearson χ^2 Goodness of Fit test for a 1:1 segregation ratio.

Missing larvae were assumed to be dead and not identifiable because of desiccation. For each population, mean larval mortalities for *cry1Ia1* positive and *cry1Ia1* negative progeny were calculated and compared using Tukey's honestly significant difference $(HSD)_{0.05}$ (JMP 9.0, SAS Institute Inc.). The same analysis was conducted on data combined across all populations.

Tuber Bioassays. A subsample of *cry1Ia1* positive and *cry1Ia1* negative lines were randomly selected for a single replication of tuber bioassays that were conducted as described by Douches et al. (2002) using greenhouse grown tubers for each clone.

Results and Discussion

Segregation Ratios. Populations MSX970, MSX971, MSX972, and MSX973 had *cry1Ia1*-positive:*cry1Ia1*-negative segregation ratios of 38:36, 38:57, 14:8, and 16:14 respectively (Fig. 1; Table 1). Southern analysis of SpuntaG2 (the female parent in all four populations) indicated the presence of a single, nuclear copy of the *cry1Ia1* gene (Zarka et al. 2010). Therefore, we would expect any population developed by crossing SpuntaG2 with a nontransgenic line to have a 1:1 segregation ratio (*cry1Ia1* positive:*cry1Ia1* negative) because of the hemizygous state of the gene. Chi-squared analysis demonstrated that the segregation ratios for each of the four populations did not differ significantly from a 1:1 model (Table 1), further supporting the previous conclusion (Zarka et al. 2010) that there is a single copy of the *cry1Ia1* gene in SpuntaG2. These results also demonstrate that there is no preferential selection either for or against embryos or seedlings carrying the *cry1Ia1* gene during the breeding process.

Efficacy of the *cry1Ia1* Gene. Progeny identified by PCR as *cry1Ia1* positive were mostly undamaged by the potato tuberworm larvae with limited feeding (damage was less than one larval body length) in a few cases (Table 2). In all four populations, the larval mortality for the *cry1Ia1* positive progeny was 100% at 72 h. This was significantly different from the *cry1Ia1* negative progeny that had mean larval mortalities of only 1–3% (Table 2). In tuber bioassays, lines with the *cry1Ia1* gene had no tuberworm damage (data not shown). These results support similar potato tuberworm susceptibility to SpuntaG2 demonstrated in leaf and tuber bioassays and field studies (Douches et al. 2010a,b; Rondon et al. 2009). Stable transmission of the *cry1Ia1* gene from SpuntaG2 to progeny with full retention of efficacy against potato tuberworm was also demonstrated.

Although certain wild potato species show some resistance to potato tuberworm (Raman and Palacios 1982, Chavez et al. 1988, Malakar and Tingey 1999) there has been no success in capturing this resistance in commercially viable clones. In contrast, SpuntaG2 has been shown to control potato tuberworm without the use of insecticides and to preserve harvested yield by minimizing tuber loss during storage (Douches et al. 2010a,b). Because of the growing global demand for potato cultivars with improved nutritional quality, pest/disease resistance, and stress tolerance, other methodologies including transgenic approaches may be necessary to achieve these goals. Our results in combination with previous reports (Douches et al. 2010a, Quemada et al. 2010, Zarka et al. 2010) demonstrate that the SpuntaG2 transgenic event controls potato tuberworm, is a stable, single copy incorporation of the *cry1Ia1* gene, with functional equivalence to Spunta. Furthermore, the transgene is transmitted in the expected Mendelian ratio (1:1) with full retention of efficacy against potato tuberworm. Thus, SpuntaG2 is a viable germplasm source from which to disseminate the *cry1Ia1* gene into important potato germplasm as well as potential production cultivars.

Table 2. Results of potato tuberworm, detached leaf bioassays in four potato populations segregating for the *cry1Ia1* gene

Family	Pedigree		Progeny		Mining	Mortality (percent)[a]
	Female	Male	*cry1Ia1* ±	No.		Mean (±SE)
X970	SpuntaG2	MSL211–3	*cry1Ia1* +	37	No	100 (0.0)a
			cry1Ia1 −	37	Yes	1.8 (0.7)b
X971	SpuntaG2	MSN105–1	*cry1Ia1* +	35	No	100 (0.0)a
			cry1Ia1 −	51	Yes	2.1 (0.5)b
X972	SpuntaG2	Silverton Russet	*cry1Ia1* +	13	No	100 (0.0)a
			cry1Ia1 −	8	Yes	3.3 (1.6)b
X973	SpuntaG2	Missaukee	*cry1Ia1* +	14	No	100 (0.0)a
			cry1Ia1 −	12	Yes	1.1 (0.8)b
Tukey $HSD_{0.05}$						1.9

[a] Means within a pedigree with the same letter are not significantly different as determined by Tukey's HSD (a = 0.05).

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