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An Analysis on Colorado potato beetle (CPB) Progenies Extreme Resistance in Somatic Hybrids of Solanum Clones

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Abstract – The Colorado potato beetle (CPB) is a major insect pest that is controlled mainly through the use of pesticides. Development of potato clones with multiple forms of host plant resistance may provide a stable alternative or supplemental form of CPB control. Tetraploid hybrids were developed by somatic fusion of diploid interspecific Solanum clones with different forms of resistance to CPB. Hybrids were created between a clone containing leptine glycoalkaloids and four clones producing glandular trichomes. One fusion produced vigorous hybrids that were analyzed for CPB resistance traits. Somaclonal variation among hybrids was detected for trichome density and resistance to feeding by adult and larval beetles. Somatic hybrids were less resistant than the parents in adult feeding preference trials, but several were more resistant than either parent in larval feeding trials. Future studies are needed to determine whether clones producing both glandular trichomes and leptines express resistance that is more stable than that of clones with only one resistance factor.

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INTRODUCTION

The Colorado potato beetle (CPB) (*Leptinotarsa decemlineata* Say) is the major defoliator of potato and, in some cases, can be the limiting factor for potato production. This insect is largely controlled by chemical means, which is expensive and potentially damaging to the environment. Furthermore, some CPB populations have developed resistance to several major classes of insecticides (Mowry and Sandvol, 1995). Utilization of host plant resistance offers a feasible alternative or supplement to chemical insect control, but is difficult because modern potato cultivars have a narrow genetic base and do not offer adequate genetic variability for selection of insect-resistant types (Sanford et al., 1984). However, sources of resistance have been found in some wild *Solanum* species.

There are two major documented sources of host plant resistance to the CPB. The first, found rarely among selections of *S. chacoense* Bitt., is production of high levels of leptine glycoalkaloids (Sinden et al., 1986a), which are effective feeding deterrents and are synthesized only in foliage (Lawson et al., 1992).

The inheritance of the ability to produce glycoalkaloids is complex because plants generally contain a mixture of glycoalkaloid types (Lawson et al., 1993). Sinden et al. (1986b) suggested, based on observations of low frequencies of high leptine clones among sibs in *S. chacoense* populations, that high levels of leptines may be produced by recessive alleles.

The second major resistance mechanism in wild *Solanum* species is glandular trichomes, which have been most thoroughly studied in *S. berthaultii* Hawkes. Gibson (1976) identified two types of glandular trichomes that are effective against insect pests. Type A trichomes are short, each with a four-lobed gland. Type B trichomes are longer, with single droplets at the tips. Pelletier and Smilowitz (1991) reported that deterrents influencing host acceptance and initiation of feeding occur in *S. berthaultii*. According to Mehlenbacher et al. (1984), heritability of droplet size of type B trichomes is high, of density of type B trichomes intermediate, and of density of type A trichomes low to intermediate. They suggested that the resistance mechanism due to trichomes is complex and should be handled as a quanti tatively inherited trait. A study by Vallejo et al. (1994a, 1994b) produced low heritability values for density of both types of trichomes and determined that additive genetic variance for trichome densities was low. Mehlenbacher et al. (1983) were able to recover parental trichome densities in F2 and backcross generations, indicating that these traits are controlled by relatively few genes. Yencho et al. (1996) have identified quantitative trait loci associated with trichome-based resistance in *S. berthaultii.*

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Solanum tarnii, a wild diploid (2*n* = 2*x* = 24), tuber-bearing Mexican species belonging to the series *Pinnatisecta* is highly resistant to Potato virus Y (PVY) and Colorado potato beetle (Thieme and Thieme 2006), and shows a strong hypersensitive reaction to *Phytophthora infestans*.

Sexual or somatic hybrids between *S. tarnii* and common potato are unknown (Jackson and Hanneman 1999). This 1EBN (Endosperm Balance Number) species is reproductively isolated from tetraploid *Solanum tuberosum* and hence diYcult to include in classical breeding programmes (Johnston et al. 1980; Jackson and Hanneman 1999). Protoplast fusion allows the transfer of both mono- and polygenic traits between sexually incompatible species. In the past, a number of attempts were made to hybridize diVerent diploid 1EBN wild species to produce somatic hybrids.

Symmetric protoplast fusion with *S. brevidens* was used to integrate virus and aphid resistance (Austin et al. 1985; Gibson et al. 1988; Valkonen et al. 1994) and tuber soft rot and early blight resistance into the potato gene pool (Polgar et al. 2000; Tek et al. 2004). Hybrids between *S. etuberosum* + *S. tuberosum* and some of their BC1 clones show increased resistance to PVY, based on mechanical inoculation (Novy and Helgeson 1994), and extreme PVY resistance, and grafting under greenhouse conditions using an aggressive isolate of PVYN (Gavrilenko et al. 2003; Thieme et al. 2004). The BC2 progeny of somatic hybrids (potato £ *S. etuberosum* + *S. tuberosum* £ *S. berthaultii* hybrids) showed an increased resistance to PVY, *Potato leafroll virus* (PLRV) and aphids in greenhouse and Weld trials (Novy et al. 2002). The PLRV resistance was transmitted and expressed in the third generation of backcrossing to cultivated potato (Novy et al. 2007). Somatic hybrids between cultivated potato and Mexican, 1EBN wild species *S. pinnatisectum* are also resistant to late blight (Ward et al. 1994; Thieme et al. 1997). Somatic hybrids between *S. bulbocastanum* and *S. tuberosum*, and backcrosses, are resistant to high exposure to late blight in the Weld (Helgeson et al. 1998). This germplasm was used in sexual crosses to transfer additional resistance into potato breeding lines. Another eYcient way of exploiting the potentially durable late blight resistance of wild *Solanum* species is to transfer these genes into existing potato cultivars by transformation. This has been described by Naess et al. (2000) and Song et al. (2003) for RB-genes and is an excellent example of the exploitation of *S. bulbocastanum* germplasm using somatic hybridization by protoplast fusion followed by gene(s) mapping, characterization and transfer by both conventional breeding and transformation.

The genus Solanum contains about 2,300 species distributed in temperate to tropical habitats, with the greatest diversity in Central and South America (Barroso et al. 1986). Among them, particularly important for the genetics and breeding of tetraploid (2n = 4x = 48) cultivated potato (S. tuberosum L.) are the approximately 200 tuber-bearing species. These belong to the subsection Potatoe, and exist as ploidy series ranging from the diploid (2n = 2x = 24) to the hexaploid (2n = 6x = 72) level. Tuber-bearing Solanum species have a wide geographic distribution, ranging from the southern part of the United States to southern Chile. Consequently, they show a very large range of ecological adaptation. For example, some species, such as S. acaule Bitter, grow in the Andean region up to 4500 m, where frost events are very common. Other wild potatoes (e.g., S. berthaultii Hawkes and S. tarijense Hawkes) are adapted to the dry semi-arid conditions of Mexico. Some wild Solanum species also have developed strong resistances to a wide range of insects and diseases, including some of the worst pests of cultivated potato such as early and late blights, viruses, potato beetle, and soft rot. By contrast, cultivated potatoes have evolved under a limited range of non-extreme environmental onditions, and are often susceptible to biotic as well as abiotic stress conditions (Hawkes 1990).

METHODOLOGY

Plant material : The potato, *S. tuberosum* L. subsp. *tuberosum* cv. Delikat (Norika, Germany), which is an early maturing variety with large oval tubers and shallow eyes, yellow skin and light yellow Xesh, was used in fusion experiments, and as a pollinator and parental cultivar in greenhouse and Weld trials.

Seeds of the diploid Mexican species *S. tarnii* Hawkes et Hjerting (Hawkes et al. 1988), accession GLKS 2870 from PK Genebank External Branch 'North', Gross Lüsewitz, Germany, were germinated in vitro, and plants from one seedling were used for propagation. The middle to late maturing cv. Sonate (Norika, Germany) was used as a standard in the Weld trials and as a pollinator for the production of BC2 progenies.

Fifteen clones belonging to two accessions, each of diploid Solanum species were screened. The wild species included two accessions (PI275190 and PI275188) of S. bulbocastanum Dunal subsp. bulbocastanum (respectively coded blb1 and blb2) and two accessions (PI 283062, PI 347759) of S. cardiophyllum Lindl. subsp. cardiophyllum (cph1 and cph2). They were provided as true seed by the IR-1 Potato Introduction Project, Sturgeon Bay, WI. Seeds for each accession were sterilized in 20% bleach for 10 min and were germinated in vitro on MS medium (Murashige and Skoog 1962), in a growth chamber (24_C and 16 h of light/day). Random seedlings from each PI were chosen and named. All studied genotypes were maintained as micropropagated plants on MS medium with 1% sucrose and 0.8% agar, and incubated at 4000 lux, 16 h light, and 24_C.

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To produce plant material for this study, four weekold-plants were transferred into styrofoam trays filled with sterile soil and acclimated to ex vivo conditions in a growth chamber at 20_C. After two weeks, they were transferred into 5-cmdiameter plastic pots and grown in a temperature-controlled greenhouse (20–24_C).

Eleven diploid (2n = 2x = 24) clones with high levels of glandular trichomes, developed by Mooney (1989), were used in fusion attempts. They were selected based on the ability to suppress development of neonate CPB larvae. The fusion partners were two high leptine diploid *S. chacoense* clones (8379-1, 8380-6), supplied by L.

Sanford (U.S. Dept. of Agriculture, Beltsville, Md.). Plants were grown in vitro on MS medium (Murashige and Skoog, 1962).

Protoplast isolation and fusion : Plants of *S. tarnii* and cv. Delikat were micro-propagated in vitro on MS5 medium (Murashige and Skoog 1962), modi Wed by reducing the NH4NO3 content to 1.2 g/l. Only one shoot was selected and analyzed for resistance, cloned and used for isolating the protoplasts of both parental species.

Shoot apices were transferred each month to MS5 medium. Three to four week old in vitro plants were used for isolating mesophyll protoplasts following the protocol of Möllers et al. (1992). The enzyme solution contained 0.2% macerozyme and 1% cellulase. After puriWcation by sequential centrifugation, parental protoplasts at 1 £ 106 pp/ml were mixed in a ratio of 1:1. This mixture was divided into 400 _l aliquots in a lamellar fusion chamber and subjected to electrofusion. The AC Weld was adjusted to a voltage of 100–200 V/cm and a frequency of 800–1,000 kHz applied for 1–2 min, 2 DC pulses of 1,200 V/cm amplitude and 15 _s duration with a brake of 2 s and a post ramp AC Weld of 10–20 s. For electrofusion, a CFA 500 device was used (Krüss GmbH, Hamburg). Fusion products and parental non-fused protoplasts were collected and cultured in modi-Wed VKM-medium with or without 2 g/l bovine serum albumin at a Wnal density of 1 £ 10 5 pp/ml and maintained at 25°C in the dark. The growing microcalluses were transferred to Cul-medium and kept under a Xuorescent light intensity of 55.5 _mol/m2/s1, a 16 h photoperiod and 25°C.

Newly formed calluses were cultured on regeneration medium RJM and kept under the same conditions. Each onth the calluses were transferred onto fresh media until the shoots developed. These shoots were rooted and propagated on MS5-medium. The media used and the corresponding references are as given by Möllers et al. (1992) and Thieme et al. (1997).

Attempts were made to isolate protoplasts from all 11 trichome clones and from the three leptine clones. The goal was to identify clones that gave sufficient viable protoplasts for fusion. For fusions using polyethylene glycol (PEG), protoplast isolation procedures were essentially those described by Haberlach et al. (1985).

The PEG fusion procedure used was essentially that of Austin et al. (1985a) and subsequent plating procedures were as described by Austin et al. (1993). Electrofusion and subsequent plating of protoplasts were performed as described by Novy and Helgeson (1994a), except that the final plating was at a concentration of 10,000 protoplasts per mL.

Crossing experiments : Flowers of greenhouse grown somatic hybrid plants were emasculated at the bud stage and pollinated with pollen of cv. Delikat to produce BC1 progeny. Cv. Sonate was used as the pollen parent for the generation of BC2 progeny. Berries were harvested and seeds cultivated in vitro using immature seeds or an embryo rescue technique (Thieme 1991). The seeds with scariWed testa were transferred to MS5-medium. In some cases embryos between the torpedo and cotyledon stages were isolated and transferred. The number of seeds plus embryos was recorded.

Statistical analysis. Trichome, glycoalkaloid, and feeding data were analyzed using the General Linear Model in SAS (SAS Institute, 1994). Duncan's multiple range test was used to compare means. Transformations of the data were performed to reduce variance heterogeneity.

Data for proportion of first instar larvae in the larval feeding analysis were arcsine transformed. Adult feeding data in 1997 were transformed by calculating the square root of the percent *S. tuberosum* leaf mass consumed.

RESULTS

Mesophyll protoplast electrofusion produced suYcient fusion products of both multi- and biparental origin. These fusion products thrived in culture, with the Wrst division occurring 3–4 days after fusion and cell colonies 1–2 mm in diameter developing in 3–4 weeks. Vigorous growth of macrocalluses and the morphology of the regenerated shoots were used as criteria for selecting the putative hybrids. In total, in two fusion trials, 3,350 calluses were cultivated. Ploidy determination allowed a more precise selection of somatic hybrids—fusion of protoplasts from diploid (*S. tarnii*) and tetraploid (*S. tuberosum* cv. Delikat) clones should produce hexaploid hybrids. In total, 63 hexaploid regenerants were selected. In addition to hexaploid plants, one mixoploid and three octoploid regenerants were also selected. Very

slightly deformed or stunted aneuploid or polyploid plants were eliminated at the in vitro stage.

Final identiWcation of interspeciWc somatic hybrids using SSR, AFLP and MFLP (not shown) analyses conWrmed the hybrid nature of 67 regenerants, showing an additive pattern of the prominent bands of the parents: the potato cv.

Results of screening diploid wild species and S. tuberosum controls for late blight resistance. Mean disease severity values ranged from 0% (10 genotypes) to 42.5% (1 genotypes). Out of nine blb clones tested, six were highly resistant, whereas three were moderately resistant, with an infection value >4.0%. The mean foliar disease severity values of S. cardiophyllum clones ranged from 15.2% (cph 2E) to 42.5% (cph 2A). Only two clones (cph 1C and cph 2D) proved to be highly resistant. These clones did not show symptoms of late blight after inoculation. On the other hand, cph 2A was the most susceptible among the evaluated clones.

DISCUSSION

The aim of this research was to enrich the cultivated potato gene pool by incorporating genes from a new exotic wild species, in order to enhance resistance to aphid transmitted PVY and late blight caused by *P. infestans*.

Sexual hybridization of diploid wild potato species from Mexico and common potato is limited because of the diVerences in ploidy levels and EBN (Johnston et al. 1980; Jackson and Hanneman 1999). Thus, protoplast fusion is the only way to introgress valuable resistant genes into the *S. tuberosum* gene pool, as it bypasses sexual incompatibility and gene segregation (Millam et al. 1995; Thieme et al. 1997, 2004; Gavrilenko et al. 2003). To improve the genetic background of potato cultivars through interspecific hybridization, the first step is the identification of sources of resistance. In this work we focused on the characterization of diploid potato germplasm with noteworthy resistances. Among all pathogens tested, P. infestans ranks beyond doubt as the world's most destructive crop disease (Garelik 2002). Indeed, breeding for resistance to light blight in potatoes was the first attempt at scientifically-based resistance breeding (Umaerus and Umaerus 1994).

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