Colorado Potato Beetle Resistance in Somatic Hybrids of Diploid Interspecific Solanum Clones

Shelley Jansky
Department of Biology, University of Wisconsin–Stevens Point, Stevens Point, WI 54481

Sandra Austin-Phillips
University of Wisconsin Biotechnology Center, Madison, WI 53706

Corine McCarthy
Department of Biology, University of Wisconsin–Stevens Point, Stevens Point, WI 54481

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.

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 supplementation 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. Gibbon (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 quantitatively inherited trait. A study by Vallesio 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 F1 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.

A major problem with control of the CPB is its genetic flexibility. Resistance to insecticides may develop quickly and has been reported widely (French et al., 1992; Herm et al., 1990; Roush and Tingey, 1991; Tsrler and Zehnder, 1990). The insect also has the potential to overcome host plant resistance. Management of transgenic plants that produce the Bacillus thuringiensis endotoxin is being carefully monitored to limit the development of insects resistant to the toxin (Everich et al., 1992; Ferro, 1993). The possibility also exists for the CPB to overcome glandular trichome-based host plant resistance (Franca et al., 1994; Groden and Casagrande, 1986).

One way to delay the development of resistant CPB populations is to combine two or more types of resistance via somatic fusion. Numerous reports exist describing somatic hybrids among Solanum species (Austin et al., 1985a; Deimling et al., 1988; Helgeson et al., 1988). Hybrids have generally been created to access novel disease resistance genes from species that are sexually incompatible with S. tuberosum L. (Barsby et al., 1984). For example, somatic hybrids have been created that are resistant to potato leaf roll virus (Austin et al., 1985b; Gibson et al., 1988), potato virus Y (Gibson et al., 1988; Novy and Helgeson, 1994a, 1994b), Phytophthora infestans (Mont.) de Bary (Helgeson et al., 1988; Menke et al., 1996), and Erwinia soft rot (Austin et al., 1988) in wild Solanum species. Gibson et al. (1988) observed intermediate levels of resistance to potato leaf roll virus and potato virus Y in somatic hybrids between a resistant and a susceptible clone.

In addition to combining genomes of incompatible species, somatic fusions can be used with sexually compatible species, such as S. berthaultii and S. chacoense. Somatic hybridization between two highly selected diploid clones may result in tetraploids with the entire genetic complement of both parents. This method, therefore, allows one to bypass normal sexual recombination and segregation. Somatic fusions between two clones with different forms of insect resistance, for example, could produce hybrids with genes for both forms of resistance. In addition, tetraploid clones so created can be crossed directly with cultivars.

Reports of somatic hybrids with insect resistance are rare. Recently, Cheng et al. (1995) reported the production of somatic hybrids between S. tuberosum and a S. chacoense clone with high levels of leptine. The somatic hybrids were not preferred as a host for the CPB, but leptine levels were
Plant material. 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. 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). If protoplasts were to be used for electrofusion, the modified isolation procedure of Novy and Helgeson (1994a) was followed. In both cases, the protoplasts were labeled with fluorescent dye after release to facilitate heterokaryon selection. After the initial centrifugation in babycock bottles (enzyme medium), protoplast bands were transferred to a 100-mL Erlenmeyer flask containing 50 mL of the appropriate rinse medium. Fluorescein diacetate (FDA) (500 mg) was added to each flask containing one set of protoplasts and rhodamine isothiocyanate (750 mg) was added to the other set of protoplasts. Flasks were placed in the dark for 1 h and then transferred to babycock bottles and centrifuged. Protoplasts were centrifuged twice more in fresh rinse medium.

Protoplast fusion and plating. 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.

Selection and regeneration of heterokaryons. Fused cells were identified 1 to 4 d after plating using dual fluorescence microscopy. Plates were viewed using a Nikon inverted microscope (Diaphot-TMD; Nikon, Inc., Garden City, N.Y.) equipped with epifluorescence. Protoplasts stained with FDA are bright green when viewed under UV light using filter casette B-2A (blue excitation 450–490 nm, barrier 520 nm). Protoplasts stained with rhodamine fluoresce bright red using filter cassette G-2A (green excitation 510–560 nm, barrier 590 nm). Heterokaryons therefore will fluoresce both green and red when viewed under the two appropriate conditions. The location of the fused cells on the plates was determined using a Nikon object marker on an inverted microscope. The marker stamps a small ink circle (diameter = 1.5 mm) on the underside of the plate. Calli appearing within these circles were picked off as they appeared (2–3 weeks later) and transferred to CUL plates as described by Haberlach et al. (1985), with t-zeatin (1.0 mg·L–1) replacing 6-benzylaminopurine (BA). When calli were light green and at least 2 mm in diameter (2–6 weeks) they were transferred to differentiation medium SA4 (MS salts with NH4NO3 at half-strength, 100 mg myoinositol, 2.5 g sucrose, 35 g mannitol, N&N vitamins (Sigma Chemicals, product #N8764), 2.0 mg t-zeatin, 0.5 mg kinetin, 0.5 mg gibberellin (GA3), 0.1 mg indole-3-acetic acid (IAA), 100 mg casein hydrolysate, and 10 g noble agar per L, filter sterilized). Any calli with shoot initials were further proliferated by transfer to a high gibberellin medium (Austin and Cassells, 1983). Shoots were excised when they were ≈1 cm in height and established on Prop medium (Haberlach et al., 1985).

Culture of hybrids. Putative hybrids, along with their parents and the cultivar Atlantic were grown in soilless mix in 15-cm-diameter pots, and fertilized with a standard timed-release fertilizer. They were placed outdoors in 1995 and grown in a greenhouse under a natural daylength in Summer 1997. These plants were used for verification of hybridity, male fertility analysis, glycoalkaloid analysis, trichome density evaluation, and CPB feeding assays.

Analysis of hybridity. Randomly amplified polymorphic DNA (RAPD) was used to verify hybridity; DNA from young leaf tissue was extracted by the method of Deragon and Landry (1992). Each 25-µL amplification reaction contained ≈25 ng template DNA in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 3.4 mM MgCl2, 0.01% gelatin, 100 µM each of dATP, dCTP, dGTP, and dTTP, 250 µM decamer oligonucleotide primer (Operon Technologies) and 1 unit AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). The following cycling parameters were used: 94 °C 0.5 min. (1 cycle); 92°C 0.5 min.; 35°C 0.5 min., 72°C 2 min. (40 cycles); 72 °C 5 min. (1 cycle). Amplification products were separated on 0.6% agarose and 1% synergel (Diversified Biotech, Newton Centre, Mass.).

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 and Discussion

Protoplast fusion and culture. There was considerable variation in the yield and viability of protoplasts from the parent clones. Overall yields were 10% to 40% of those expected from cultivated *S. tuberosum* cultivars such as Katahdin. Only one of the leptine clones (K) consistently produced viable protoplasts in sufficient quantity to use in fusion studies and also gave protoplasts that were capable of regeneration into whole plants. Of the 11 trichome clones, eight yielded protoplasts, and of these, six regenerated plants after subsequent tissue culture. Fluorescent labeling typically reduced viability by 10% to 50% in the trichome clones, but had no effect on the viability of the leptine clone.

Initial attempts to produce somatic hybrids via PEG fusion were not successful. The clones did not survive the fusion procedure well and viability of protoplasts dropped to essentially zero over the next 3 to 7 d. Electroporation was more successful. Survival rates after fusion were 45% to 60% for the trichome clones and 60% to 70% for the leptine clone. Putative somatic hybrids were produced from four high trichome clones combined with the same leptine clone (K). Typically one of 150 to 250 viable cells was dual labeled 1 to 3 d after fusion. These cells were circled as described above and any resulting microcalli were cultured further. In most experiments two to 12 microcalli were recovered from each plate. Subsequent regeneration from these calli was dependent on the trichome clone used in the study, and ranged from 2% to 18%. Many of the regenerated plants from selected calli were not vigorous compared to protoclones from parental lines. About 50% failed to root successfully either during initial establishment in culture or through subsequent subculturing.

Somatic fusions were obtained with four of the 11 high trichome clones attempted. Their pedigrees are outlined in Table 1. Only one of the leptine-producing *S. chacoense* clones (8380-6 from PI 458310) successfully produced somatic hybrids. It will be identified in this paper as clone "K." The best pair of clones for somatic hybrid production, based on number of fusion clones produced and vigor of those clones, was the high trichome clone J with the leptine clone K (Table 2). In fact, most clones from other fusions lacked sufficient vigor to be used for replicated trials. Most of the J × K fusions were vigorous, so these clones are described in this paper. Rokka et al. (1994) and Deimling et al. (1988) also reported differences in success rates among fusion partners.

Analysis of hybridity. Five of the six primers surveyed (Z10, Z12, Z13, Z18, and Z19) produced parent-specific RAPD bands. Primer Z7 did not produce unique bands. Primers Z13 and Z18 were chosen to verify hybridity because they consistently produced bands that were unique to each parent and easily scored. Twenty-eight putative hybrids were surveyed and all produced the bands of both parents. Therefore, the process of selecting for hybrids during the production of somatic fusions was effective. Several published reports have also verified somatic fusions through DNA analysis (Baird et al., 1992; Menke et al., 1996; Provan et al., 1996; Rokka et al., 1994; Takemori et al., 1994; Xu et al., 1993). This method is especially valuable because small amounts of leaf tissue are required, and hybrids can therefore be identified at an early stage in development.

Pollen stainability. Parents J and K were highly fertile, but pollen stainability was markedly reduced in the fusion hybrids (Table 3). Reduced fertility is common in somatic hybrids (Rokka et al., 1994). Chromosome aberrations, especially aneuploidy, have been reported in protoplast-derived potato plants (Karp et al., 1982). Therefore, some sterility may be due to structural chromosome changes or aneuploidy induced by protoplast culture. Eight of 18 clones exhibited low to moderate, and occasionally high pollen stainability. However, Ehlenfeldt and Helgeson (1987) reported a poor correlation between pollen stainability and crossability of tetraploid *Solanum* hybrids.
trichome parent (J10) had a significantly higher type A trichome density than did any hybrid or the leptine parent (Table 5). One hybrid, KJ62, had a higher trichome density than all other clones except J10. Twenty-nine clones had low type A densities and did not differ statistically from the leptine parent. Narrow-sense heritability and, therefore, additive genetic variance for type A trichome density, has been reported to be low (Vallejo et al., 1994a). In this study, somatic hybrids exhibited moderate to low type A trichome densities compared with the high trichrome parent. Therefore, the alleles controlling trichome density appear to be acting in a more dominant than additive fashion. All hybrids were derived from the same two clones. Therefore, assuming that both parental genomes were transmitted intact, somaclonal variation would account for differences in type A trichome density (Table 5).

This type of variation in protoplast-derived potato plants has been reported extensively (Belknap et al., 1994; Karp et al., 1982; Shepard, 1981; Shepard and Totten, 1977). Scowcroft and Larkin (1982) suggest that somaclonal variation may be caused by epigenetic variations, karyotypic changes, mutations in nuclear and chloroplast DNA, chromosomal changes, and non-conventional mutations, such as gene amplification or transposable elements. Williams et al. (1993) provided evidence for deletions in somatic hybrids. Al- though somaclonal variation has been reported for vine, flower and tuber traits, along with numerous cases of gene amplification or transposable elements. This type of variation in protoplast-derived potato plants has been reported extensively (Belknap et al., 1994; Karp et al., 1982; Shepard, 1981; Shepard and Totten, 1977). Scowcroft and Larkin (1982) suggest that somaclonal variation may be caused by epigenetic variations, karyotypic changes, mutations in nuclear and cytoplasmic DNA, chromosomal changes, and non-conventional mutations, such as gene amplification or transposable elements. Williams et al. (1993) provided evidence for deletions in S. brevidens Phil. + S. tuberosum somatic hybrids. Although somaclonal variation has been reported for vine, flower and tuber traits, along with disease resistance, it has not been documented for trichome traits in potato.

Significant differences were also observed among clones, but not replications, for density of type B trichomes (Table 4). The high trichome parent (J10) contained a high density of type B trichomes (85.33 trichomes/25 mm²). However, no type B trichomes were observed in the other parent or any of the hybrids. Therefore, ANOVA could not be performed when parents were excluded. Previous reports indicate that density of type B trichomes is complexly inherited, with low levels of additive genetic variance (Meihlenbacher et al., 1984; Vallejo et al., 1994a). Our results indicate that the inheritance of type B trichomes may be recessive, since these trichomes were not observed in any somatic hybrids. Alternatively, cytoplasmic DNA may affect the expression of type B trichomes, as suggested by Vallejo et al. (1994b). Somatic hybrids presumably contain equal quantities of mitochondrial and chloroplast DNA from both parents, in contrast with sexual hybrids. Therefore, the S. chacoense cytoplasm in the somatic hybrids may have inhibited the expression of genes for type B trichomes.

Glycoalkaloid analysis. Although the S. chacoense clone K produced leptines, their levels were low. Analysis of variance revealed no significant difference among clones for total glycoalkaloids, solanidine (solanine and chaconine glycoalkaloids), leptinidine (leptine glycoalkaloids), and acetylleptinidine (leptine glycoalkaloids), and acetylleptinidine (leptine glycoalkaloids).
Adult beetles always preferred leaves of *S. tuberosum* to those of the parents or the somatic hybrids. However, no somatic hybrids were more resistant than both parents. Clones that were among the most resistant included KJ74, KJ69, and KJ81. Note that clones varied in resistance to feeding by adult beetles even though they did not vary in glycoalkaloid levels. In addition, the somatic hybrid with the highest level of type A trichomes was not among the most resistant clones, and the most resistant clones did not differ significantly from less resistant ones in type A trichome density. Some other resistance mechanism must be influencing the acceptance of the somatic hybrid leaves by adult beetles. The contents of the trichomes may vary among clones. An alternative explanation may be suggested by the study of Yencho et al. (1996), in which a strong and consistent quantitative trait locus was reported for CPB resistance that was not associated with any trichome traits in *S. berthaultii* hybrids.

The larval trial focused on first instar larvae because they would presumably be most affected by chemically-based resistance mechanisms. In fact, few clones except *tbr* allowed development of larvae past the second instar. According to Dimock and Tingeny (1987), large larvae tend to accumulate exudate on more of their tarsi than do young larvae and are, therefore, more likely to be affected by the mechanical impediments conferred by glandular trichome-based resistance. These observations indicate that the major resistance factor affecting larvae in these somatic hybrids is chemically-based and present in either glandular trichomes or leaf tissue.

Several somatic hybrids inhibited larval development significantly more effectively than one or both parents (Table 7). The greatest number of differences between clones and parents were observed in 1997. As discussed previously, trichomes may have been damaged more extensively in 1995, when the clones were grown outdoors. Chemical resistance factors in the trichomes may have been responsible for the greater resistance of clones vs. their parents in 1997. Clone KJ57 was more resistant than both of its parents in both years of trials.

Several clones were more resistant than either parent in the larval feeding trials. These clones may be expressing resistance mechanisms from both parents, enhancing their ability to inhibit larval development. Alternatively, they may have the potential to express heterosis more extensively than do their diploid parents. Another factor may be the opportunity for novel recombination products because cytoplasmic DNA from both parents is present in the somatic fusions (Kumar and Cocking, 1987). Finally, the resistance mechanisms that impede larval development may be expressed more effectively at the tetraploid than at the diploid level. Because these hybrids have not been challenged with CPBs for several generations, it is not known whether their resistance is more durable than that of the parents. Future research could help elucidate the long-term effectiveness of resistance factors in the somatic hybrids.

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