Potato Transformation to Combine Natural and Engineered Resistance for Controlling Tuber Moth

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Additional index words: genetic engineering, lepilina, eyeclick, glandular trichome, Bacillus thuringiensis, Phloxtheuma operculata, Solanum tuberosum

Abstract: Potato tuber moth (Phloxtheuma operculata Zeller) is a highly destructive pest of the cultivated potato (Solanum tuberosum L.) in the tropics and subtropics and causes significant damage to both leaves and tubers. Development of host plant resistance is a central component of an integrated pest management (IPM) program for potato tuber moth. The purpose of this research was to augment natural resistance by transforming potato with a codon-modified Cry V-B gene cooperating with Agrobacterium-mediated techniques. 'Lenhsl Russet' potato and two clones with different host plant resistance mechanisms, USDA5880-1 (leaf lesions) and L235-4 (glandular trichomes), were transformed with the Cry V-B gene. Gene integration of regenerated plants was confirmed by polymerase chain reactions and Southern analyses; gene transcription was evaluated by northern analyses. Detached leaf bioassays showed that high levels of Bt expression occurred in the Cry V-B transgenic lines ('Lenhsl Russet' and L235-4), providing up to 96% control of potato tuber moth larvae, compared with 3% and 54% control in L235-4 and USDA5880-1, respectively. These transgenic lines can be used for breeding purposes to develop cultivars for (and eventual introduction into) IPM systems.

One of the most important insect pests of the cultivated potato is the potato tuber moth, which causes damage to plants in the field and to tubers in storage in tropical and subtropical regions. Potato tuber moth larvae attack the potato by mining in the leaves and tubers (Traver and Ragupati, 1992). Larvae tunnel and feed on the leaves, veins, and stems of the plant, causing loss of leaf tissue, death of growing points, and weakening or breaking of the stems (Raman, 1980). The tubers are infested by larvae emerging from eggs deposited on the surface of the soil near the stem, in cracks in the soil, and near the eyes of stored tubers. The larvae mine into the tuber, causing irregular tunnels near the scar of the leaf as well as inside the tuber. This mining introduces new organisms and renders the tubers unfit for human consumption.

Host plant resistance is a potential component of an integrated pest management program to control the tuber moth. Two types of host plant resistance have been identified in potato: high levels of leaf glycolycauloids (Sinden et al., 1986) and glandular trichomes (Plaister et al., 1992). Introduced Bacillus thuringiensis (Bt) toxins genes offer an additional source of insect resistance. These Bt genes have been cloned, codon-modified, and inserted into various crop species (Barton and Miller, 1990). The efficacy of codon-modified Bt genes, such as Cry1Ac and Cry1B, is greater than that of wild-type Bt genes when inserted into crop plants (Petrik et al., 1990, Wenz et al., 1996). The Cry V-B gene we used in this study is effective against both Lepidoptera and Coleoptera and has also been codon-modified to increase its expression in plants (J. Tippett, personal communication).

The overall objective of this research was to use Agrobacterium-mediated transformation to combine the codon-modified Cry V-B gene with natural mechanisms of resistance in the potato tuber moth. The specific research objectives were to: 1) obtain transgenic plants expressing the Cry V-B toxin gene, and 2) document gene integration and expression via Southern analyses, northern analyses, and detached leaf bioassays.
BREEDING, CULTIVARS, ROOTSTOCKS, & GERMPLASM RESOURCES

Fig. 1. Diagram of the DNA region of the plant hBry-V. This gene construct was made by ligating the 2.2 kb Bry-V-B fragment into the BamII site adjacent to the CaMV 35S promoter within pBI121 (ChuaNing, Pali-Apte, et al.).

find using a mini-fluorometer model TKO100 (Labsystems Scientific). Five P. furvus. Calif. DNA was amplified according to Gilco BRL (Bolzaedoa, Md.) with the Cyv-B1 (forward 5'-AAC TGG AAG TCG GTG GTG GTG GGC GTG GCA ATA CCA A3'); reverse 5'-GCA TCG CCC TGC TCC CCC TCC CTC GCT G3') primers producing 684 bp and 255 bp fragments, respectively. The amplification conditions were: 1 cycle = 4 min at 94°C; 40 cycles = 1 min at 94°C, 1 min at 50°C, 1.5 min at 72°C. Once completed, a 30-μl aliquot of the polymerase chain reaction (PCR) reaction mix was separated on a 1% agarose gel and stained with ethidium bromide (0.5 μg/ml). Plants that had >80% potato tuber moth mortality in bioassays (Wuestedt et al., 1990) were also analyzed by Southern blot for gene integration and copy number. Total DNA was extracted according to the method of Saghai-Maroof et al. (1986), modified by using 2% beta-mercaptoethanol in the extraction buffer. The DNA (10 μg) from each line was digested with BamHI to cut out the Cyv-B gene, and the copy number was analyzed by digesting with XhoI. The fragments were separated by electrophoresis through a 1.0% agarose gel, dried onto a nylon membrane (Hybond N, Amersham, England) using a capillary transfer procedure (Sambrook et al., 1989). The membranes were acidified to the membrane by a cross-linking solution (10% acetic acid, 50% ethanol, 0.1% sodium metaperiodate, 30% formamide, and 125 μl shed salmon sperm DNA. Hyridization was performed at 42°C overnight in a solution with a non-radioactive, random primed DIG labeled probe (2.2 kb BamHI fragment containing the Cyv-B gene). The probe was labeled using the Direct-Prime (Stratagene) kit. Hybridization and stringency were amplified by PCR using the 373T primer according to manufacturer's instructions (Genset 2 Kit, BMB, Indianapolis). Following hybridization, the membrane was washed twice in 2× SSC plus 0.1% SDS for 15 min at room temperature, and twice in 0.5× SSC plus 0.1% SDS for 20 min at 65°C. To detect chemiluminescence 0.25 μg disorder 4-3 methoxy poly(L,2-dio- diate 3.2 M-chloroacetoxy 3,3-dimethyl-3-buten-1-ol) plus phosphate (30°), Toprix Inc., Bedford, Mass. was added according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, 1995). The membranes were then exposed to X-ray film (Kodak XOMAT, Rochester, N.Y.) for 15 to 30 min. Total RNA from a young leaf was isolated by using the Qiagen RNeasy Plant Total RNA Kit (Qiagen, Chatsworth, Calif.). Total RNA (20 μg) was fractionated by formaldehyde gel electrophoresis through a 1.0% agarose gel in MOPS buffer (20× MOPS, 5× MOPS sodium acetate, 1× EDTA, 0.5% acetic acid, pH 4.0), followed by blotting onto a Hybond N nylon membrane. Hybridization, hybridization, and hybridization conditions were as described above for DNA analysis, except that the blots were hybridized at 52°C and a Cyv-B RNA probe was used.9 synthesizing in an in vitro run-on transcription of the Cyv-B DNA in a BlueScript Sk+ vector (Stratagene) according to manufacturer's instructions (Ge- nius 4 Kit, BMB).

Results

Plant transformation with Cyv-B. Pote- tive transgenic shoots were first propagated on 'Lemhi Russet' explants for 4 weeks and shoot production continued for 2 months. A total of 40 individual shoots were removed and transferred to kanamycin rooting medium (50 mg/l). The first 33 shoots to root in this medium were then used to determine the presence of the Cyv-B gene. A total of 27 of these 33 lines were PCR-positive for the Cyv-B gene. Nontransgenic plants often developed rooted roots, but these roots only grew on the medium surface and up the shaft of the culture tube. In contrast, transgenic lines produced more quickly and the roots sprouted through the medium. Clone USDA58801 was slower to gener- ate shoots, which emerged from 8 to 12 weeks after transformation. However, once emerged, they grew rapidly in the kanamycin rooting medium. Initially, 60 shoots were se- lected, of which 30 rooted. All but one of these rooted plants were PCR-positive for Cyv-B. Line L23-2-5 was the most diffe- rent type from which to obtain transgenic plants. Using twice as many leaf disks for L23-2-5 as for 'Lemhi Russet', 45 healthy shoots were obtained for the rooting assay. Shoot regener- ation occurred after 8 to 12 weeks. Fifteen shoots rooted in the kanamycin rooting me- dium and 14 of these were PCR-positive for the Cyv-B gene.

Potato tuber moth bioassays. Eighteen PCR-positive 'Lemhi Russet' Cyv-B and nontransgenic 'Lemhi Russet' lines were used in potato tuber moth bioassays. Mortality of the moths ranged from 20% to 95% among the transgenic 'Lemhi Russet' lines, with all transgenic lines producing higher mortality than the nontransgenic lines (data not shown). Mortality on the eight most resistant 'Lemhi Russet' lines was 85% to 93%, significantly higher than on the control leaves (Table 1).

Twenty-five transgenic lines of USDA58801-Cry-B were tested. The nontransgenic USDA5830-1 line had 24% mor- tality in larvae. A wide range of potato tuber mortalities were observed among larvae on the USDA58801-Cry-B lines (40% to 100%) (data not shown). Larvae on the seven most resistant USDA58801-Cry-B lines Table 1. Mortality of potato tuber moth larvae on foliage of three cultivars of potato and Cyv-B transgenic lines in potato tuber bioassays.

<table>
<thead>
<tr>
<th>Line</th>
<th>Mortality (%)</th>
<th>Leaf</th>
<th>Line</th>
<th>Mortality (%)</th>
</tr>
</thead>
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<tr>
<td>'Lemhi Russet'</td>
<td>70</td>
<td>47</td>
<td>USDA58801</td>
<td>85</td>
</tr>
<tr>
<td>Lemb-1</td>
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<td>27</td>
<td>8500-1-1</td>
<td>75</td>
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<td>88</td>
<td>39</td>
<td>8500-1-26</td>
<td>75</td>
</tr>
</tbody>
</table>

2% value significantly different from the nontransgenic lines at P<0.05. Wilcoxon test.

Three replications per treatment.

Eight replications per treatment.

Four replications per treatment.

Our purpose was to combine natural host plant resistance mechanisms with the Cry-VB gene via transformation, thus mimicking the best plant resistance mechanisms against potato tuber moth. The Agrobacterium tumefaciens-mediated transformation procedures to obtain transgenic plants. This transformation protocol produced transgenic lines for all potato genotypes. The highest efficiency was in genotypes, with 90% of all potato genotypes. The optimal genotype-dependent responses to their regeneration protocol and did not re-

Niewoehner et al. (1991) proposed a transformation technique using potato stamen sections. Transgenic plant recovery was 2% to 5%. Our transgenic plant recovery was at the 30% to 50% level; however, these numbers may be biased since double-shoots were observed in other studies. Some somaclonal variation was observed among the regenerated plants. Minor distortion of leaf morphology was observed. Some somaclonal variation from the regeneration process may also affect the subsequently grown resistance factors, lepidopteran, and glandular tri-

The expression and leaf levels among the transgenic lines would resolve this dilemma. Western blot analysis of Crys-Bt protein levels and leaf levels (Sinden, 1987), respectively, would apply. The vector construct used in this study had the B-glucosidase (GS5) gene fused to the Crys-Bt gene, with the CaMV 35S promoter controlling both genes (Fig. 1). The results of the Crys-Bt transgenic plants demonstrated that high levels of expression of the Crys-Bt gene could be obtained. Similarly, southern blot analysis showed that the Gus gene was also maintained in these Crys-Bt transgenic lines. Comparison of the expression of Crys-Bt constructs in potato with and without the Gus gene would be of interest. The biossay results from the Crys-Bt transgenic lines of 'Len shiny Russet' and L235-

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4 demonstrated that high levels of Cry-V-bit expression can be achieved with this gene construct. However, insect pests don’t adapt to survive Bt toxins applied as foliar insecticides or injected into plants (Whalon et al., 1993). If Bt expression is to be used as a tool in host plant resistance management strategies, then the next step is to look at how the lines with combined resistances can be used in a potato/tuber moth management strategy. Both the glandular trichomes and epidermis may affect the moth at the host acceptance stage (Sindrirotta et al., 1986; Yenipha and Teigey, 1994). Under field conditions, these natural host resistance mechanisms may reduce the number of moth eggs landing on the plant as a host and ovipositing. This would reduce selection for adaptation to Cry-Vb in lines with combined resistance mechanisms. Even if adult behavior and oviposition are not affected, the availability of multiple resistance factors can be used in combination or sequentially to manage potato/tuber moth adaptation to host plant resistance factors. The insect bioassay used in these studies demonstrated high levels of Bt expression in the leaf; however, the tuber is the other economically damaged organ of the potato plant. The next key step is to determine whether potato/tuber moth larvae are controlled by Cry-Vb in the tubers. The constitutive expression of the cry55 promoter, was used in our Cry-Vb construct (Kuzy and Chiue, 1990b) (Fig. 1). Bioassays to determine if expression in the tuber is correlated with that in leaves will be of value. In addition, field studies will compare the value of host plant resistance on potato tuber moth among these Cry-Vb-gemotypes. Our next step is to test the Cry-Vb gene in combination with the Galvia superpromoter (Narushima et al., 1996) and the potato promoter (Weinrich et al., 1989) for expression levels.

Literature Cited