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Genetic engineering of potato for resistance to tuber moth

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1 Executive summary

A collection of potato lines transgenic for *cry* genes known to confer resistance to potato tuber moth larvae have been evaluated in a contained field trial. A small-scale field trial involving 10 plants in 3 m plots for each of 92 transgenic potato lines was planted in early December 1999. These lines included 42 Iwa lines and 5 Red Rascal lines transgenic for an improved version of the *cry*9Aa2 gene, none of which had been previously field tested. Also included were 28 Iwa and 17 Red Rascal lines transgenic for either the *cry*1Ac gene or earlier versions of the *cry*9Aa2 gene that had been previously field tested over the 1997/98 and/or the 1998/99 seasons.

Bioassays against larvae were performed on foliage from 69 of the transgenic lines. Forty-four Iwa lines (81%) and nine Red Rascal lines (60%) exhibited improved resistance to potato tuber moth larvae in the field. Transgenic lines identified in the 1997/98 and 1998/99 field trials as having high resistance against the tuber moth larvae continued to exhibit a high degree of resistance in the 1999/00 field trial. The growth of potato tuber moth larvae significantly reduced in virtually all of the lines that were transgenic for the improved version of the *cry*9Aa2 gene and that had not previously been field tested.

Statistical analysis established that the tuber yields of 35 of the 70 transgenic Iwa lines and 13 of the 22 transgenic Red Rascal lines were not significantly less than the non-transgenic control lines for all three yield parameters measured (number of tubers per plants, total weight of tubers per plant, and weight of individual tubers).

Repeated field evaluation of the Iwa and Red Rascal lines transgenic for the *cry*1Ac gene that exhibited high peformance in the 1997/98 and/or the 1998/99 field trials, continued to perform in a similar manner in the 1999/00 field trial. Several new transgenic lines from each of Red Rascal and Iwa, expressing a new improved version of the *cry*9Aa2 gene, had high field resistance to potato tuber moth larvae and yield potential that did not differ from the parental cultivars. These lines are suitable candidates for future scale-up field trials.

Results of the 1999/00 field trial confirm that high performing potato cultivars that are transgenic for* cry *genes remain stable from season to season, confirming the value of genetic engineering for resistance to potato tuber moth larvae.

2 Introduction

Potato tuber moth is the major insect pest of potato crops in New Zealand. It is especially prevalent in dry seasons, and is a field and postharvest problem. The resulting physical damage to potato tubers makes them unmarketable and can also result in the development of secondary disease infections. Current control methods for potato tuber moth in potatoes include cultural management practices, biological control and the use of broad spectrum

insecticides. Some cultural practices, such as planting time, planting depth, irrigation, re-moulding and hilling, pre-harvest removal of foliage with herbicide applications, crop hygiene and immediate removal of tubers to cold storage facilities, assist in reducing damage by potato tuber moth larvae. However, heavy infestations of potato tuber moth still require the use of repeated applications of organo-phosphates, synthetic pyrethroid, and/or carbamates for effective control.

The use of genetic engineering to clone *cry* genes from *Bacillus thuringiensis*, and to transfer and express these genes in plants to confer resistance to insects is well established. This approach is currently being attempted in many crops throughout the world to develop resistant cultivars against specific insect pests. New Zealand potato cultivars with *cry* genes for resistance to potato tuber moth that have been produced by genetic engineering may be an important component of integrated pest management systems against this pest. Such cultivars will assist in reducing the reliance on pesticides to control this insect pest and produce high quality tubers without insect damage and with a minimum of pesticide residues.

From the previous research funded by FRST, Crop & Food Research and the Potato Industry Research & Development Committee, a large collection of transgenic potato lines already exists for a range of important cultivars in New Zealand. These transgenic lines are transformed with either a *cry*1Ac gene (already shown to be effective in our previous field trials - see Conner et al. 1999) and a new improved version of a *cry*9A2 gene (no material has been previously field tested with this new gene).

The objective of this project was to contribute to a small scale field trial and identify valuable transgenic potato lines by:

- evaluating at least 40 potato lines transgenic for the *cry*9A2 gene,
- performing bioassays on field-grown foliage, and
- assessing plant appearance and yield performance.

3 Experimental methods

3.1 Gene transfer to potato

The general methods for gene transfer to potato are outlined in Conner et al. (1996). Leaf segments from *in vitro* cultured potato plants were dipped in cultures of *Agrobacterium* strains containing binary vectors with *cry* genes. The *Agrobacterium* was cultured with the plant tissue for two days to allow gene transfer to take place, then eliminated by incorporating specific antibiotics into the culture medium. The presence of a gene conferring kanamycin resistance to plant cells on the same vector as the *cry* gene permitted transformed cells to be selected by incorporating kanamycin into the culture medium. Sequential changes in the content of plant growth regulators in the culture media allowed the growth and regeneration of the transformed cells into complete plants (Conner et al. 1991).

Each independently derived transgenic shoot was clonally multiplied via micropropagation, transferred to a containment greenhouse and hardened-off using our standard procedures (Conner et al. 1994). The resulting plants were used to establish the field trial.

3.2 Field trial

Rows of the potato plants were spaced 75 cm apart, with 30 cm between plants within rows. Ten plants of each line were transplanted as a single plot within a row, with a 1 m gap between plots. The trial involved the planting of 70 Iwa and 22 Red Rascal lines transgenic for *cry* genes. Plots of these transgenic lines were interspersed with plots of the appropriate non-transgenic control—18 of Iwa and 6 of Red Rascal. The experimental plots were completely surrounded by plots from trials on other transgenic potatoes or 3 buffer rows of non-transgenic potato to prevent "edge effects" during the trial. The buffer rows were planted with a potato genotype that had purple tubers to allow them to be readily distinguished from the white or pink-red tubers of the transgenic potato lines.

Throughout the trial observations were made on the general appearance of the transgenic lines. At various times leaves were excised and used for feeding bioassays with larvae of potato tuber moth. At harvest the number of tubers and weight of tubers per plot were recorded.

3.3 Tuber moth bioassays

The growth of potato tuber moth larvae feeding on the field grown plants was determined on excised leaves on moistened filter paper in 250 ml plastic containers. Five leaves were placed in each container with five pre-weighed neonate larvae. The larvae were transferred to fresh leaves after five days. Three replicate containers for each line were maintained at room temperature for 10 days, after which the larvae were individually reweighed. A growth index (GI) of the the recovered larvae was calculated as:

GI = ln final weight - ln initial weight.

This index provides a measure of the mean relative growth of the larvae. The frequency of larval mortality during the experiment was also recorded.

3.4 Statistical analysis

Bioassay and yield data were examined with REML methods (Welham 1993). These methods allow for the removal of any field trends, and the varying numbers of plots, pottles and larvae per pottle, or plants per plot, for each line. As part of the analysis each line was compared with the relevant non-transgenic control line.

4 Results

Negotiations with the Environmental Risk Management Authority (ERMA) allowed the relocation of our trial site to a new site at an undisclosed location. This was considered necessary following the vandalism of our field trials last year. It meant some new criteria requested by ERMA had to be met.

The transgenic potato lines were multiplied *in vitro* during September-October 1999. Twelve plants from each line were transferred to a containment greenhouse in mid-October, then further hardened-off in a screen house. These plants were transferred to the trial site and established in the field in early December 1999. The trial included 42 Iwa and 5 Red Rascal lines transgenic for the improved version of the *cry*9Aa2 gene, none of which had been previously field tested. Also included were 28 Iwa and 17 Red Rascal lines transgenic for either the *cry*1Ac gene or earlier versions of the *cry*9Aa2 gene that had been previously field tested over the 1997/98 and/or the 1998/99 seasons. Repeated testing of these lines allowed the stability of performance in potato lines transgenic for *cry* genes to be assessed, and also provided an important baseline for cross-referencing the relative performance of the new transgenic lines not previously field tested.

The survival and establishment of the potato transplants derived from tissue culture was 90% for both the Iwa and Red Rascal plants. The majority of the losses were associated with specific transgenic lines (Iwa lines T24, T33, T54, T73, TG1, TG2, TG3, TG4, and TG5; Red Rascal lines T12, T64, and T74). Some plots in certain parts of the trial exhibited poor growth of the potato plants. This was attributed to patches of poor drainage over the trial site shortly after the establishment of the trial. The planting of numerous non-transgenic plots interspersed among the transgenic lines (one in every five plots) allowed appropriate *a posteriori* blocking and correction factors to be made to the data.

Sixteen of the 70 transgenic Iwa lines and 7 of the 22 transgenic Red Rascal lines exhibited marked changes in the appearance of the foliage in comparison to the non-transgenic controls. In general this involved reduced plant vigour, often associated with puckered and/or malformed leaves. Such changes in appearance are commonly observed in potato plants regenerated from tissue culture and are not unexpected in populations of transgenic plants (Conner et al. 1994). These lines were not used for bioassays against tuber moth larvae since any inhibition of insect growth could be due to factors others than the expression of the *cry* genes.

Bioassays against larvae of potato tuber moth were performed on foliage from the remaining 54 transgenic Iwa lines and 15 transgenic Red Rascal lines. Statistical analysis of the bioassay data using REML established that lines could be compared using the variation between the plastic containers for each line (ignoring the original field plots). The analysis indicated that 44 Iwa lines (81%) and 9 (60%) Red Rascal lines exhibited significantly reduced growth of the potato tuber moth larvae that survived on their leaves (Table 1). The growth indices for some of the remaining lines were also low, but were not significantly different to the non-transformed controls due to high error terms and a low sample size of surviving larvae. These included five Iwa lines (02, 74, 75, 76, and D48) and four Red Rascal lines (D02, D20, D23, and D53), all of which exhibited statistically significant field resistance to

potato tuber moth larvae over the 1998/99 field season (Conner et al. 1999). Despite the non-significance of larval growth indices on these lines over the 1999/00 field season, larvae mortality was substantially higher on these lines than on the control lines (Table 1). Bioassays were performed on 31 of the 47 lines transgenic for the improved version of the *cry*9Aa2 gene that had not been previously field tested. Both of the Red Rascal lines and 26 of the 29 Iwa lines exhibited significantly reduced growth of the potato tuber moth larvae (Table 1).

The tuber yield data from the individual transgenic and control lines are presented in Table 2. Statistical analysis established that the yields of 35 of the 70 transgenic Iwa lines and 13 of the 22 transgenic Red Rascal lines were not significantly less than the non-transgenic control lines for all three yield parameters measured (number of tubers per plants, total weight of tubers per plant, and weight of individual tubers). These included 20 of the 47 lines with the improved *cry*9Aa2 gene not previously field tested. For the lines that had been previously evaluated in the field, their yield performance was similar to that recorded in 1997/98 (Davidson et al. 1998) and/or 1998/99 (Conner et al. 1999) relative to the non-transgenic controls.

Table 1: Summary of resistance of field-grown transgenic potato lines to potato tuber moth larvae. All lines prefexed DG, SI, TG and SR are transgenic for the improved cry*9Aa2 gene and had not been previously field tested.*

Cultivar and line	*cry* gene	Mortality (%)	Mean larval growth index of survivors
Iwa (batch 1)			
Control	-	4	4.12
02	9Aa2	8	2.90
32	9Aa2	42	1.94*
41	9Aa2	46	1.41*
43	9Aa2	17	2.31*
52	1Ac	71	0.76*
74	9Aa2	29	3.20
75	1Ac	54	2.58
76	9Aa2	13	3.32
92	9Aa2	38	2.00*
D18	1Ac	50	1.20*
D48	1Ac	29	2.82
D54	1Ac	58	2.21*
LSD[a]	Min	-	0.91
	Max	-	3.31
	Mean	-	1.79
Iwa (batch 2)			
Control	-	40	5.55
T18	1Ac	40	2.82*
T20	1Ac	33	3.54*
T24	1Ac	67	3.39*
T33	1Ac	47	3.86*
T48	1Ac	40	3.22*
T49	1Ac	40	3.59*

Cultivar and line	*cry* gene	Mortality (%)	Mean larval growth index of survivors
T50	1Ac	40	4.15*
T51	1Ac	40	3.47*
T54	1Ac	33	5.42
T68	1Ac	33	5.24
T69	1Ac	40	4.82*
T70	1Ac	40	4.96*
T73	1Ac	60	4.59*
DG1a	9Aa2	40	4.24*
DG1h	9Aa2	67	3.77*
DG3a	9Aa2	73	3.67*
DG3b	9Aa2	67	4.28*
DG3c	9Aa2	53	3.88*
DG4c	9Aa2	60	3.90*
DG4d	9Aa2	67	4.81*
DG4e	9Aa2	60	4.03*
DG5b	9Aa2	53	3.99*
SI3a	9Aa2	60	4.03*
SI3b	9Aa2	53	4.08*
SI5a	9Aa2	53	4.15*
SI6a	9Aa2	47	4.27*
SI9d	9Aa2	53	4.51*
SI9e	9Aa2	60	4.16*
SI9g	9Aa2	47	3.93*
SI10a	9Aa2	67	4.01*
SI10b	9Aa2	80	3.72*
SI12b	9Aa2	53	4.23*
SI13a	9Aa2	53	4.04*
SI24b	9Aa2	67	3.91*
SI25c	9Aa2	60	4.34*
SI26a	9Aa2	53	4.61*
SI28b	9Aa2	73	3.86*
TG1	9Aa2	40	5.14
TG2	9Aa2	60	3.93*
TG3	9Aa2	47	5.20
TG4	9Aa2	53	3.98*
TG5	9Aa2	40	5.31
LSD[a]	Min	-	0.04
	Max	-	0.65
	Mean	-	0.46
Red Rascal (batch 1)			
Control	-	0	2.62
D02	1Ac	87	1.15
D20	1Ac	60	1.79
D23	1Ac	60	0.74
D53	1Ac	87	0.43
LSD[a]	Min	-	2.26
	Max	-	6.19
	Mean	-	3.83
Red Rascal (batch 2)			
Control	-	33	5.52
T09	1Ac	53	3.23*
T11	1Ac	47	3.82*
T35	1Ac	60	5.72
T65	1Ac	47	4.12*
T74	1Ac	40	4.00*
T75	1Ac	67	4.10*

Cultivar and line	*cry* gene	Mortality (%)	Mean larval growth index of survivors
T76	1Ac	53	3.79*
T78	1Ac	40	3.73*
T86	1Ac	60	5.24
SR4b	9Aa2	53	3.87*
SR6a	9Aa2	47	4.51*
LSD[a]	Min	-	0.42
	Max	-	0.53
	Mean	-	0.45

[a]Least Significant Difference at the 5% probability level to compare any transgenic line with the non-transgenic control. Because the number of pottles containing larvae varied between lines, the LSD varies for each cultivar. Consequently, the smallest (Min), the largest (Max), and the mean LSD for each batch are presented.

Note: Of the 92 transgenic potato lines, 16 Iwa lines (D47, T22, T53, G3d, G3e, G3f, G4a, G4b, G4f, SI6b, SI9a, SI9c, SI9f, SI14a, SI24a, SI28a) and 7 Red Rascal lines (SR1a, SR1b, SR4a, T12, T13, T62, T64) were not tested in bioassays against potato tuber moth due to marked phenotypic changes in the appearance of the field-grown foliage such as reduced plant vigour and puckered or malformed leaves.

Table 2: Summary of the yield data from field-grown transgenic potato lines. The lines in bold are those whose yield was not significantly less than the non-transgenic control line for all three yield parameters. All lines prefexed DG, SI, TG and SR are transgenic for the improved cry*9Aa2 gene and had not been previously field tested.*

Cultivar and line	Number of tubers per plant	Weight of tubers per plant (g)	Mean weight per tuber (g)
Iwa			
Control	**7**	**358**	**54**
02	5	129	35
32	3	89	34
41	4	310	92
43	**6**	**846**	**148**
52	**4**	**104**	**27**
74	3	405	88
75	**10**	**786**	**73**
76	3	214	60
92	2	126	61
D18	5	173	35
D47[a]	**9**	**474**	**56**
D48	**5**	**207**	**47**
D54	**5**	**234**	**44**
T18	**10**	**237**	**26**
T20	3	134	42
T22[a]	4	9	3
T24	2	15	6
T33	7	213	20
T48	**22**	**1111**	**49**
T49	**4**	**132**	**37**
T50	**13**	**331**	**26**
T51	**8**	**258**	**26**
T53[a]	**10**	**946**	**87**
T54	**15**	**922**	**53**
T68	**12**	**439**	**38**
T69	**10**	**473**	**49**
T70	**6**	**209**	**35**

Cultivar and line	Number of tubers per plant	Weight of tubers per plant (g)	Mean weight per tuber (g)
T73	**14**	**420**	**29**
DG1a	**8**	**487**	**59**
DG1h	**7**	**228**	**37**
DG3a	12	262	25
DG3b	**12**	**262**	**25**
DG3c	16	290	22
DG3d[a]	**6**	**129**	**23**
DG3e[a]	6	177	31
DG3f[a]	**9**	**392**	**32**
DG4a[a]	6	151	23
DG4b[a]	7	84	12
DG4c	**12**	**1277**	**92**
DG4d	**13**	**843**	**65**
DG4e	**22**	**1511**	**70**
DG4f[a]	5	83	17
DG5b	3	70	23
SI3a	6	149	31
SI3b	7	93	17
SI5a	9	170	24
SI6a	5	172	32
SI6b[a]	**4**	**116**	**30**
SI9a[a]	**10**	**253**	**26**
SI9c[a]	**11**	**566**	**41**
SI9d	**7**	**399**	**50**
SI9e	**7**	**372**	**50**
SI9f[a]	5	92	21
SI9g	**11**	**438**	**43**
SI10a	3	77	35
SI10b	8	95	13
SI12b	7	154	26
SI13a	**7**	**266**	**37**
SI14a[a]	3	57	17
SI24a[a]	3	54	17
SI24b	8	194	26
SI25c	**6**	**285**	**45**
SI26a	6	178	29
SI28a[a]	3	62	18
SI28b	3	56	19
TG1	4	114	36
TG2	3	101	48
TG3	**10**	**512**	**63**
TG4	**6**	**304**	**48**
TG5	6	153	32
LSR[a]			
Min	1	2	2
Max	3	7	3
Mean	2	2	2
Red Rascal			
Control	**7**	**112**	**18**
D02	4	58	18
D20	3	88	42
D23	**6**	**77**	**15**
D53	**7**	**145**	**21**
T09	**11**	**176**	**16**
T11	**13**	**225**	**17**
T12[a]	**6**	**123**	**17**
T13[a]	**5**	**84**	**18**
T35	**8**	**193**	**22**

Cultivar and line	Number of tubers per plant	Weight of tubers per plant (g)	Mean weight per tuber (g)
T62[a]	4	62	17
T64[a]	2	30	17
T65	5	41	10
T74	3	33	15
T75	**9**	**100**	**13**
T76	**8**	**227**	**27**
T78	**7**	**91**	**13**
T86	**15**	**493**	**25**
SR1a[a]	6	53	8
SR1b[a]	4	48	11
SR4a[a]	2	41	14
SR4b	**4**	**96**	**22**
SR6a	**5**	**95**	**19**
LSR[a]			
Min	1	2	2
Max	3	7	3
Mean	2	3	3

[a]Lines exhibiting marked phenotypic changes in the appearance of the field-grown foliage such as stunted plants, reduced plant vigour and/or leaf puckering.

[b]Least Significant Ratio for comparing transgenic lines with the control. If the ratio is greater than this, then the mean is significantly different from the control at the 5% probability level. The minimum, maximum and mean LSRs for the data are provided because each possible comparison requires a different LSR due to the varying number of plants harvested for each line.

Note: Means presented were obtained from analysis of the log of the data, corrected for the waterlogged plots and a trend across the rows of the trial. Means are for numbers or weights assuming all plots were dry. The weight of tubers per plant as presented is not equivalent to weight/number due to transformation of the data and the corrections applied.

5 *Discussion*

The results of this study further establish the value of genetic engineering for developing transgenic potato lines with improved resistance to larvae of potato tuber moth. The repeated field evaluation of 28 Iwa lines and 17 Red Rascal lines transgenic for either the *cry*1Ac gene or earlier versions of the *cry*9Aa2 gene showed a similar performance from year-to-year with respect to both bioassays for resistance to larvae of potato tuber moth and tuber yield. This is an important finding as it confirms that transgenic lines can perform in a stable manner from season to season.

The main aim of this field trial was to evaluate the performance of 42 Iwa lines and 5 Red Rascal lines transgenic for the improved version of the *cry*9Aa2 gene that had not been previously field tested. A high level of resistance to larvae of potato tuber moth was exhibited by 90% of these lines, with 43% giving tuber yields not significantly less than the control lines. We have, therefore, identified a second *cry* gene, in addition to our previous success with the *cry*1Ac gene, that can provide effective field resistance to potato tuber moth. This will substantially enhance our ability to develop management plans for the release of genetically engineered potatoes with the expectation of minimal risk that the insect pest will overcome the resistance mechanism.

When genetic engineering clonal crops, such as potato, the aim is to recover a transgenic line with the desired new character while retaining all of the elite genetic attributes of the parent cultivar (Conner & Christey 1994). Independently derived transgenic lines can vary markedly in the effectiveness of the transferred gene. Furthermore, since the transgenic lines have been regenerated from tissue culture, the appearance of off-types is not uncommon. The main reason for evaluating numerous potato lines transgenic for the same *cry* gene in the same cultivar is, therefore, to identify the few elite lines with both a high level of resistance to the insect pest and all the desirable characteristics of the parent cultivar. For each potato cultivar the aim is to identify one-two lines transgenic for each *cry* gene as suitable candidates for future scale-up field trials.

For the *cry*1Ac gene we had previously identified Iwa line 52 and Red Rascal lines D02 and D53 as candidates for scale-up field trials (Davidson et al. 1998; Conner et al. 1999). The results of the 1999/00 trial have confirmed the value of these lines, as well as identifying Iwa line T48 and Red Rascal lines T09 and T76 as potential candidates. For the *cry*9Aa2 gene the most promising lines include DG1a and DG4c for Iwa and SR4b for Red Rascal.

6 References

Conner, A.J.; Christey, M.C. 1994: Plant breeding and seed marketing options for the introduction of transgenic insect-resistant crops. *Biocontrol Science and Technology 4:* 463-473.

Conner, A.J.; Davidson, M.; Reader, J.; Takla, M.; Butler, R. 1999: Genetic engineering of potato for resistance to tuber moth. *CropInfo Confidential Report No. 650*. 16 p.

Conner, A.J.; Lambie, S.C.; Perera, S.; Reader, J.K.; Shum-Thomas, T.; Genet, R.A.; Wigley, P.J. 1996: Genetic engineering of the potato for insect resistance. *Commercial Grower 51(8):* 22-26.

Conner, A.J.; Williams, M.K.; Abernethy, D.J.; Fletcher, P.J.; Genet, R.A. 1994: Field testing of transgenic potatoes. *New Zealand Journal of Crop and Horticultural Science 22:* 361-371.

Conner, A.J.; Williams, M.K.; Gardner, R.C.; Deroles, S.C.; Shaw, M.L.; Lancaster, J.E. 1991: *Agrobacterium*-mediated transformation of New Zealand potato cultivars. *New Zealand Journal of Crop and Horticultural Science 19:* 1-8.

Davidson, M.; Reader, J.; Takla, M.; Conner, T. 1998: Genetic engineering for potato tuber moth resistance. *CropInfo Confidential Report No. 515.* 9 p.

Welham, S. 1993: REML estimation of variance components and the analysis of unbalanced designs. Chapter 3. In: *Genstat 5 release 3, reference manual*. Genstat committee, OUP.