Theor Appl Genet (1997) 95: 1062–1068

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A secondary effect of transformation in *Rhizobium leguminosarum* transgenic for *Bacillus thuringiensis* subspecies *tenebrionis* δ-endotoxin (*cryIIIA*) genes

Received: 20 April 1997 / Accepted: 2 June 1997

Abstract By introducing *Bacillus thuringiensis* subspecies *tenebrionis* δ-endotoxin genes (*cryIIIA*) into *Rhizobium leguminosarum* we have produced strains for the biological control of *Sitona* larvae. Comparisons between a transgenic and the parent strain show that transformation has induced changes not associated with the intended function of the transgene. Although growth rates in laboratory cultures are similar for both strains, the ability to compete for nodule occupancy is greater in the transgenic than in the non-transformed parent strain. This result demonstrates the importance of studying ecological and agronomic characters of transgenic micro-organisms that could have a bearing on the safety and success of their release into the environment, even if they are not thought to be connected with the transgenes introduced.

Key words *Rhizobium* · Transformation · Nodule occupancy

Introduction

Molecular methods of introducing foreign DNA into *Rhizobium* offer the potential for enhancing the commercial impact of inoculants (Bezdicek et al. 1994). We have previously reported the results of introducing *cryIIIA* genes into *Rhizobium* (Skøt et al. 1990). The transgenic strains produce a polypeptide which is toxic to various coleopteran insects including *Sitona* species. The larvae of *Sitona* feed on legume roots and nodules and can cause considerable damage (Witty et al. 1980; Quinn and Hower 1986; Brown and Gange 1990; Bezdicek et al. 1994). Some of the transformed strains confer a degree of biological control to this important pest (Skøt et al. 1994) but the environmental impact of transgenics is of wide concern and it is essential to ensure that there are no unacceptable consequences associated with releasing them into the environment (Stotzky and Babich 1985; Shorrocks and Coates 1993).

The primary effects of transgenes for agriculturally important traits will, of course, be well characterised. These effects may vary according to genetic background and much is now known about the potential for gene transfer between transgenics and wild relatives (e.g. Stotzky and Babich 1985; Ellstrand and Hoffman 1990; Dale 1992). Another, less-well-considered possibility, is that of secondary effects of transformation leading to altered ecological or agronomic properties of the transgenic compared with non-transformed counterparts. Of particular concern are traits that affect the ability of transgenics to persist and spread once released, i.e. characters affecting fitness (Tiedje et al. 1989).

Theoretically, secondary effects associated with transformation might occur due to the transgenic construct causing an insertion mutation, particularly as transgenes are often inserted into the genome at random (de Bruijn and Lumpski 1984; Dale et al. 1993). This could have pleiotropic effects, or there may be pleiotropic effects of the transgene itself, in the absence of mutation (Dale et al. 1993). Such pleiotropy is common in bacterial mutants selected for novel phenotypes and is usually maladaptive, leading to reduced fitness (Lenski 1988 a). It is often assumed that the secondary effects of transformation will cause similar reductions in fitness making the transgenics less of a risk in the environment (Regal 1988; Dale et al. 1993; Raybould and Gray 1993; Williamson and Fitter 1996). Beringer and Bale (1988) concluded that since genetically

Communicated by P. M. A. Tigerstedt

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engineered micro-organisms (GEMs) have only one or a few specified genes added or deleted, and differed from the parent by about 1 in 2000 genes, they should, compared to the parent strain, "… behave in the same way once released into the environment, unless the added gene, or genes, modify the ecological properties of the strain."

Few studies have been conducted on the fitness of transgenics (Kareiva 1993). Where fitness has been examined generally no difference has been observed between transgenics and non-transformants. Experiments on an engineered strain of *Pseudomonas aureofaciens*, for example, showed that it was as effective as the parent strain in establishing large populations on inoculated wheat-plant roots (Drahos et al. 1988). Similarly, no differences were found in the growth rates of recombinant Ice^- *Pseudomonas syringae* and the Ice^+ parental strains, including those obtained after co-inoculation of potato plants with equal numbers of each strain (Lindow et al. 1988). Nevertheless Kareiva (1993) concluded that the lack of evidence for differences in fitness between transgenics and their parent strains is probably as much a result of the lack of appropriate experimentation as a reflection of the true situation.

The need for more information on the fitness and competitive ability of transgenics is widely acknowledged (e.g. Liang et al. 1982; Drahos et al. 1988; Tiedje et al. 1989; Kareiva 1993; Cresswell et al. 1994). In the present report we have made comparisons between an agriculturally important strain of *Rhizobium leguminosarum* and a transformed derivative containing *Bacillus thuringiensis* subspecies *tenebrionis* (B.t.t.) toxin genes. We have considered two characters which have the potential to influence competitive ability and fitness. These are, ability to multiply and ability to compete for nodule sites on pea plants.

Materials and methods

Rhizobium Strains

The strains used were described in detail by Skøt et al. (1990, 1994). The parent (LS2202) is a strain of a *R. leguminosarum* biovar *viciae* resistant to streptomycin. The transgenic (LS2238) contains the *B. thuringiensis* subsp. *tenebrionis cryIIIA* gene, as well as kanamycin and gentamycin resistance genes, integrated into the chromosome. The promoter responds to legume root exudates and gives enhanced levels of toxin expression in the rhizosphere.

Rhizobium Culture

Rhizobium strains were cultured under sterile conditions in 250-ml conical flasks containing 100 ml of tryptone yeast broth (TY, Beringer 1974) aerated on an orbital shaker at 15°C. Parent cultures were diluted in yeast mannitol broth (Vincent 1970), counted and standardised to 10^6 viable cells per ml. Experimental cultures were seeded with aliquots of diluted standardised parental cultures.

Rhizobium population density

The viable organisms of each culture were estimated using a drop-count procedure (Mytton and de Felice 1977). Ten-fold serial dilutions were made using sterile water. Dilutions ranged between 10^0 and 10^{-7} depending on the turbidity of the culture, as judged from experience.

Ten 2-µl aliquots were taken from each serial dilution and dropped onto the dried surface of yeast mannitol-agar plates containing congo red dye (YMCR, Vincent 1970). Plates were inverted and incubated for 2 days at 15°C. The number of developing colonies in each drop were counted using a binocular microscope (×120 magnification). The assumption was made that each colony represents a single viable cell.

Plant Culture

Pea plants (*Pisum sativa*) were grown axenically, from surface-sterilised seed, in 12.5-cm diameter plastic pots using the method of El-Sherbeeny et al. (1977). The rooting medium was washed vermiculite. Seedlings received an initial addition of 100 ml of sterile Jensen's nitrogen-free nutrient solution (Vincent 1970); thereafter sterile distilled water or nutrient solution was added as required. Plants were grown in a controlled environment room set to give a 16-h photoperiod with quantum irradiance from daylight fluorescent tubes plus incandescent lamps of approximately 300 $\mu mol\, m^{-2} s^{-1}$ and a day/night temperature regime of 20/10°C or 25/20°C.

Rhizobium inoculants

Inoculum was prepared as previously described (Mytton and de Felice 1977). Cultures were grown to approximately 10^7 viable cells ml^{-1}. These were then standardised to 10^6 ml^{-1} by dilution. Mixed inoculum was prepared by shaking cultures together for 15 min on a wrist action shaker to give the ratios required at cell densities of 10^6 ml^{-1}.

Isolation of rhizobia from nodules and strain identification

Rhizobium isolates were obtained by surface-sterilising nodules for 1 min in 30% (v/v) sodium hypochlorite followed by several rinses in sterile distilled water. Individual nodules were crushed with a sterile glass rod and the contents streaked onto pairs of YMCR agar plates, one containing appropriate antibiotics and one without (Vincent 1970). Concentrations of antibiotics for strain identification were 200 $\mu g\, ml^{-1}$ of streptomycin for LS2202 and 50 $\mu g\, ml^{-1}$ of kanamycin for LS2238. Antibiotic agar was prepared as described by Mytton and de Felice (1977). Our previous experiments indicate that nodules typically result from infection by a single rhizobium under the conditions employed in these experiments.

Model fitting

Genstat (version 5.3; Genstat 5 Committee 1987) and MLP (version 3.09; Ross 1987) were used for statistical analysis and curve fitting. The error structure was investigated to seek a transformation, and weights to homogenise and normalise residuals. The growth curves fitted were in the form of a generalised logistic:

$$y = a + \frac{c}{(1 + te^{-b(x-m)})^{(1/t)}},$$

in which m is the point of inflection, i.e. the value of x at which growth is maximum, a is the lower asymptote as $x \to -\infty$ and $(a + c)$ is the upper asymptote as $x \to \infty$. t reflects the asymmetry of the curve and the pattern of decrease in relative growth rate: if $t = 1$ then the curve is the standard symmetric logistic with a linear decline in relative growth rate; as $t \to 0$ the curve tends towards a Gompertz curve with an exponential decline in relative growth rate; as $t \to \infty$ the curve tends towards a "sudden arrest shape" with a constant relative growth rate, i.e. exponential growth until $y = a + c$ and zero growth thereafter. For a given value of t, b reflects the relative rate of approach to the upper asymptote: at the point of inflection when $x = m$, the relative growth rate is $b/(1 + t)$.

Curves were fitted to each replication of each strain, to each strain ignoring replication and to the pooled data, ignoring strain and replication. The goodness of fit of the strain curves was tested using:

$$F_{good} = \frac{(RSS_{strain} - RSS_{replication})/(df_{strain} - df_{replication})}{MSS_{replication}},$$

where RSS and MSS are the residual and mean sums of squares and df the degrees of freedom. The numerator is the MSS for the difference between fitting strains and fitting replications individually. F_{good} has $(df_{strain} - df_{replication})$ and $df_{replication}$ degrees of freedom. A similar test was used to determine the goodness of fit of a single curve for all data compared with fitting one curve to each strain. Thus an anova was constructed to determine whether there are differences in growth between strains and between replications within strains.

Experimental

Population growth

Aliquots (100 ml) were taken from bulked standardised mother cultures and dispensed into 250-ml conical flasks containing 200 ml of TY broth (four replicates of LS2238 and three of LS2202). Cultures were grown for 13 days under the conditions described above. Population density was estimated every 24 h based on ten drop counts from each replicate.

Competition for nodule sites

Five experiments were carried out to determine the nodule occupancy of pea plants (cultivar Meteor) after inoculation with a 1:1 ratio of transgenic: parent strains, two with the peas growing at warm temperature (25/20°C) and three at normal temperature (20/10°C). In a sixth experiment was with a 10 parent: 1 transgenic mixture at normal temperature. A final experiment was conducted using three alternative pea cultivars: Onward, Senator and Little Marvel.

Seven-day-old pea seedlings were inoculated with 32 ml of the appropriate inoculum and cultured as above. Replicate plants for each inoculation treatment were arranged in a randomised design (4–8 replicates per experiment). Plants were harvested after 30 days and a sample of nodules removed for strain isolation and identification.

Results

Population growth in culture

Error structure

The data are weighted according to dilution, since counts become increasingly less accurate with increasing dilution. Weights are $1/10^n$ where n is the number of 1-in-10 dilutions. A square-root transformation with weights, while not completely homogenising the errors, reduces $\chi^2_{homogeneity}$ by more than 100-fold (from 18834.7 to 179.8) and eliminates skewness (from 0.67 to -0.02).

Fitting the growth curves

Table 1 shows the anova testing the goodness of fit of curves with common intercepts (a, displacement), linear parameters (c), and non-linear parameters (b, m, t).

Table 1 Analysis of variance of growth between replications within strains and between strains

Source of variation	Sums of squares	Degrees of freedom	Mean square	Variance ratio	Probability
Between replications within LS2202					
Displacement	0.53	2	0.26	0.94	> 0.05
Common linear	0.00036	2	0.00018	0.001	> 0.05
Common non-linear	0.60	6	0.10	0.35	> 0.05
Within data sets	6.79	24	0.28		
Between replications within LS2238					
Displacement	0.12	3	0.04	0.22	> 0.05
Common linear	0.61	3	0.20	1.09	> 0.05
Common non-linear	1.27	9	0.14	0.75	> 0.05
Within data sets	6.01	32	0.19		
Between strains					
Displacement	12.88	1	12.88	64.65	< 0.01
Common linear	0.17	1	0.17	0.85	> 0.05
Common non-linear	0.07	3	0.02	0.12	> 0.05
Within data sets	16.13	81	0.20		

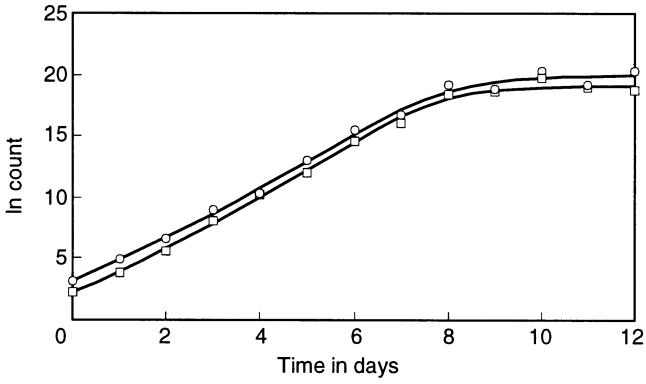

Fig. 1 Mean growth rate shown as ln count over time in days in LS2202 (*circles*) and LS2238 (*squares*). Fitted generalised logistic growth curves shown as *solid lines*

There are no significant differences between replicates for the growth curves of either LS2202 or LS2238, indicating that a common curve can be used for each. The only significant difference between strains is in the displacement, indicating a difference in the size of starting population. The dynamics of growth are the same for each strain, with both strains reaching their maximum growth rate on day 5 (Fig. 1).

Competition for nodule sites

Inoculation of P. sativa cultivar Meteor with 1:1 parent:transgenic mixtures

From Tables 2 and 3 it can be seen that more root nodules are occupied by the transgenic than the parent strain after inoculation of cultivar Meteor with 1:1 mixtures of parent:transgenic rhizobia at both temperature treatments in 5 separate experiments.

Table 4 Chi-square test for heterogeneity between replicates and deviation from a 1:1 parent:transgenic nodule-occupancy ratio

Source of variation	χ^2	*d.f.*	*P*
Experiments 1 to 3 at normal temperature			
Deviation	78.09	1	< 0.001
Heterogeneity	22.41	14	> 0.05
Experiments 1 to 3 at warm temperature			
Deviation	12.17	1	< 0.001
Heterogeneity	5.90	8	> 0.05

Table 4 shows that there is no between-replicate heterogeneity at either temperature, and that the overall deviation from a 1:1 ratio is highly significant in both cases. The observed ratio is 2.4 transgenics: 1 parent at the normal temperature treatment and 1.9:1 at the higher temperature treatment, indicating that the transgenic competes more successfully for nodule sites than the parent.

Inoculation of P. sativa cultivar Meteor with 10:1 parent:transgenic mixtures

More root nodules are occupied by the transgenic than expected if there were no change from the inoculation ratio of 10 parent:1 transgenic (Table 5). There is no between-replicate heterogeneity and the overall deviation from a 10:1 ratio is highly significant (Table 6). The observed ratio is 10 parent:2.7 transgenic. The results are consistent with those from the previous experiments and support the observation that the transgenic competes more successfully for nodule sites than the parent.

Table 2 Occupancy of nodules of *P. sativa* cultivar Meteor grown at normal temperature after inoculation with 1:1 mixtures of parent:transgenic rhizobia

Nodule occupancy	Experiment 1 1995						Experiment 2 1995						Experiment 3 1996				Total counts
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
Transgenic	36	39	39	26	42	30	40	8	9	11	13	9	7	7	10	12	338
Parent	14	11	11	24	8	20	10	7	6	4	2	6	3	3	5	8	142
Totals	50	50	50	50	50	50	50	15	15	15	15	15	10	10	15	20	480

Table 3 Occupancy of nodules of *P. sativa* cultivar Meteor grown at warm temperature after inoculation with 1:1 mixtures of parent:transgenic rhizobia

Nodule occupancy	Experiment 4 1995					Experiment 5 1996					Total counts
	1	2	3	4	5	6	7	8	9	10	
Transgenic	8	10	10	11	9	6	9	8	5	6	82
Parent	7	5	5	4	6	4	1	2	5	4	43
Totals	15	15	15	15	15	10	10	10	10	10	125

Table 5 Occupancy of nodules of *Pisum sativa* cultivar Meteor grown at normal temperature after inoculation with 10:1 mixtures of parent:transgenic rhizobia. Expected occupancy shown in brackets

Nodule Occupancy	Experiment 6					Total counts
	1	2	3	4	5	
Transgenic	16 (5.46)	13 (5.46)	12 (5.46)	14 (6.82)	12 (5.46)	67
Parent	44 (54.55)	47 (54.55)	48 (54.55)	61 (68.18)	48 (54.55)	248
Totals	60	60	60	75	60	315

Table 6 Chi-square test for heterogeneity between replicates of experiment 6 and deviation from a 10:1 parent:transgenic nodule-occupancy ratio

Source of variation	χ^2	*d.f.*	*P*
Deviation	107.79	1	< 0.001
Heterogeneity	5.41	3	> 0.05

Inoculation of P. sativa cultivars Onward, Senator and Little Marvel with 1:1 *parent:transgenic mixtures*

From Table 7 it can be seen that more root nodules are occupied by the transgenic than the parent strain after inoculation of pea cultivars Onward, Senator and Little Marvel with 1:1 mixtures of parent:transgenic rhizobia. Table 8 shows that there is no between-replicate heterogeneity in each case. The overall deviation from a 1:1 ratio is highly significant for Senator and Little Marvel, but not for Onward. The observed ratios of transgenic:parent are 1.48:1 for Onward, 3.35:1 for Senator, and 2.8:1 for Little Marvel. The transgenics ability to compete for nodule sites is significantly better than the parents on three of the four pea genotypes tested.

Comparison of variation between experiments

Figure 2 shows the mean number of transgenic rhizobia recovered from root nodules per transgenic expected if there had been no change from the ratio in the inoculum, with the line indicating that expected. As there is no heterogeneity within experiments a test was done to determine whether there is heterogeneity between experiments in deviation from expected ratios. The results in Table 9 show that there is significant heterogeneity between experiments even when the results for occupancy of pea cultivar Onward are left out on the basis of their difference from expectation being insignificant. Nevertheless, if we leave out the data from experiment 6, with the 1 transgenic:10 parent inoculation mixture, there is no heterogeneity regardless of whether the Onward results are included in the analysis. When the parent is initially in excess the mean number of transgenic rhizobia recovered from nodules per transgenic expected is significantly greater than that observed for 1:1 inoculation mixtures. The mean ratio of transgenic:parent for nodules inoculated with a 1:1 mixture is 2.3:1, and for nodules inoculated with a 1:10 mixture 2.7:10.

Table 7 Occupancy of nodules of *Pisum sativa* cultivars Onward, Senator and Little Meteor grown at normal temperature after inoculation with 1:1 mixtures of parent:transgenic rhizobia

Nodule occupancy	1	2	3	4	5	6	Total counts
Onward 1996							
Transgenic	12	7	5	6	8	5	43
Parent	3	3	5	4	9	5	29
Totals	15	10	10	10	17	10	72
Senator 1996							
Transgenic	8	12	15	7	7	8	57
Parent	2	5	2	3	3	2	17
Totals	10	17	17	10	10	10	74
Little Marvel 1996							
Transgenic	8	14	16	14	11	7	70
Parent	2	6	2	6	6	3	25
Totals	10	20	18	20	17	10	95

Table 8 Chi-square test for deviation from a 1:1 parent:transgenic nodule-occupancy ratio and heterogeneity between replicates taken from nodules of the cultivars Onward, Senator and Little Meteor

Source of variation	χ^2	*d.f.*	*P*
Onward			
Deviation	2.72	1	> 0.05
Heterogeneity	4.74	4	> 0.05
Senator			
Deviation	21.62	1	< 0.001
Heterogeneity	1.60	4	> 0.05
Little Meteor			
Deviation	21.32	1	< 0.001
Heterogeneity	2.64	4	> 0.05

Summary of results

(1) There were no significant differences between replicates within any of the experiments.
(2) No difference was observed in the population dynamics of LS2202 and LS2238 in laboratory culture.
(3) For all experiments there were more nodules occupied by the transgenic strain than expected if there had

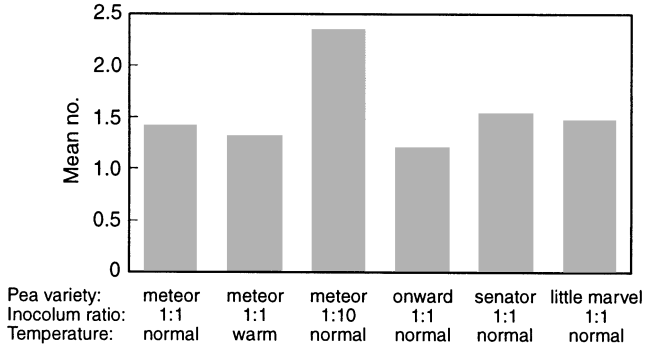

Fig. 2 Mean number of transgenics recovered from nodules per transgenic in the inoculum

Table 9 Chi-square test for deviation from expected nodule-occupancy ratios and between experiment heterogeneity

Source of variation	χ^2	*d.f.*	*P*
All experiments			
Deviation from expectation	152.83	1	< 0.001
Heterogeneity	41.56	4	< 0.001
Without results from Onward			
Deviation from expectation	153.10	1	< 0.001
Heterogeneity	38.57	3	< 0.001
Without results from experiment 6			
Deviation from expectation	131.86	1	< 0.001
Heterogeneity	6.00	3	> 0.01

been no change from the ratio of transgenic to parent in the initial inoculation mixture. The increase was significant for two temperature regimes, for 1:1 and 1:10 inoculation mixtures, and for nodules on pea varieties Meteor, Little Marvel, and Senator but not on variety Onward.

(4) There is no significant difference in the mean number of transgenic rhizobia recovered from nodules per transgenic expected for experiments where a 1:1 inoculum was used. A mean ratio of 2.3 transgenic:1 parent was observed.

(5) When a 1:10 inoculum was used the mean number of transgenic rhizobia recovered from nodules per transgenic expected is significantly greater than that observed for 1:1 inoculation mixtures. A mean ratio of 2.7 transgenic:10 parent was observed.

Discussion

Transformation has induced a change the transgenic rhizobium that unrelated to the function of the transgenes inserted. Greater-than-expected nodule occupancy indicates that the engineered strain better able to compete for nodule sites than the parent strain, although the B.t.t. toxin transgene was inserted to confer pest resistance of nodulated pea roots. This character has remained stable over 2 years of experiments.

The mechanism of the increased ability to compete for nodule sites is unknown. The population dynamics were observed to be the same for the parent and transgenic, so it is not simply a result of the transgenic's ability to grow faster. Furthermore, when inoculation mixtures were used with the parent strain in excess the deviation from expectation was significantly greater than when 1:1 mixtures were used indicating that a mechanism is operating independently of simple density dependence. It is, however, possible that plant-rhizobium or transgenic-parent interactions induce differential growth in the rhizosphere or affect the nodulation process itself. On one pea variety the transgenic did not form significantly more nodules than expected, which may be evidence of a genotype-specific plant-rhizobium interaction. However, there is no significant heterogeneity between the experiments using different pea-plant varieties so this result should be viewed with caution until more data are available.

The effect observed may be due to pleiotropic effects of the transgene itself or to the positional affect of the Tn5 transposon that carries the gene. New transconjugants of LS2202, which are likely to harbour the transgenic construct in a different position of the genome (de Bruijn and Lumpski 1984), have been made to test the possibilities. Experiments are also being conducted to determine whether the reported effects can be observed in soil ecosystems; initial results seem to indicate that this is so.

Regardless of the explanation, an enhanced ability to compete for nodule sites would be a desirable feature of inocula (Cresswell et al. 1994; Skøt et al. 1990). Strains with this feature may be more likely to associate with the crop and less likely to form free-living colonies, which could be more hazardous to the environment. It might also be possible to use lower levels of inoculum to achieve the same nodulation rates, thus reducing the exposure of the environment overall.

It would be surprising if we had, by chance, picked the only strain of the only organism in which there are secondary effects of transformation. In fact recent evidence indicates that some transgenic oilseed rape exhibits pleiotropic effects which may positively affect fitness in some environments (Linder and Schmitt 1995). Furthermore, the fact that manganese superoxide dismutase genes have pleiotropic effects is being put to use in the development of cold-tolerant transgenic alfalfa. This gene confers protection against cold damage and also tolerance to herbicides, which is being used both as a marker and for selection (Bridger et al. 1994; McKersie et al. 1996).

Since secondary effects of transformation are possible the importance of studying the fitness and other (e.g. productivity) characteristics of transgenics prior to their release should not be under-estimated. Differences in function between transgenics and non-transformants

could have a bearing on the safety and success of releases. Considering the concern over the safety of releasing genetically modified organisms into the environment this is something well worth investigating further. In mutants, adverse effects of pleiotropy on fitness are often ameliorated by selection for epistatic modifiers (Clarke and McKenzie 1987; Lenski 1988 b). Those planning to release transgenics should not forget that evolution will act to increase their fitness once they are released, at which time secondary effects of transformation may become important (Tiedje et al. 1989; Jones 1995).

Acknowledgements A special thanks to Deborah Allen and David Hughes who provided technical assistance. Thanks also to Dr. Ruaraidh Sackville Hamilton who gave advice on the statistical analysis of the growth-rate experiment.

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