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A secondary effect of transformation in *Rhizobium leguminosarum* transgenic for *Bacillus thuringiensis* subspecies *tenebrionis* δ-endotoxin (*cryIIIA*) genes. Part 2

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Summary *Rhizobium leguminosarum* strains were produced for the biological control of *Sitona* larvae by introducing *Bacillus thuringiensis* subspecies *tenebrionis* delta-endotoxin genes (cryIIIA). Comparisons between a transgenic and 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 sites is greater in the transgenic than in the non-transformed parent strain, a character that has remained stable over 4 years. This increased ability, which was previously observed in axenic culture, is shown here to also occur in non-sterile soil, although the effect is less pronounced than in sterile conditions. Experiments in soil show a highly significant difference from the expected nodule occupancy ratio, assuming no difference between genotypes and with no significant variation between replicates. These results demonstrate that the ecological and agronomic characters of transgenics might be unexpectedly altered by transformation. Such characters might have a bearing on the safety and/ or success of transgenics released into the environment.

Key words *Rhizobium* · Transformation · Nodule occupancy · Soil

Introduction

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). *Rhizobium leguminosarum* strains were produced for the biological control of these coleopteran insects by introducing *Bacillus thuringiensis* subspecies *tenebrionis* δ-endotoxin genes (*cryIIIA*; Skøt et al., 1990, 1994).

We have previously demonstrated that transformation induced a change in one of the transformed strains that is not associated with the intended function of the transgene (Giddings et al. 1997). Although growth rates in laboratory cultures are similar for transformed and parent strains the transgenic has an increased ability to compete for nodule sites, a character that has remained stable over 4 years. These results demonstrate that the ecological and agronomic performance of transgenics could be unexpectedly altered by transformation. This might have a bearing on the safety and/ or success of transgenics released into the environment. The 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. Our previously published data was derived from axenic culture experiments in artificial media. The experiment presented here extends the study to the more realistic environment of the soil.

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Materials and methods

Rhizobium strains

The strains employed 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 and a kanamycin resistance gene, integrated into the chromosome. The promoter responds to legume root exudates and gives enhanced levels of toxin expression in the rhizosphere.

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 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}.

Soil preparation

Soil from the Plas-y-mynydd (Angler's Retreat) region of West Wales (Ordnance Survey reference: 746924) was used in this experiment as it has previously been found to be free of native pea *Rhizobia*. This was confirmed by the absence of nodules on peas grown in samples of the soil prior to the experiment. $CaCO_3$ was used to adjust the pH of the soil to 7.

Plant culture

Pea plants (*Pisum sativa* cv Meteor) 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 µmol m^{-2} s^{-1} and a day/night temperature regime of 20/10°C.

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 µg ml^{-1} streptomycin for LS2202 and 50 µg ml^{-1} kanamycin for LS2238. Antibiotic agar was prepared as previously described (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.

Competition for nodule sites

Seven-day-old pea seedlings were inoculated with 32 ml of inoculum and cultured as above. Three plants were used per replicate, with 25 replicates arranged in a randomised design. Controls were seven plants inoculated with each strain individually, and seven plants uninoculated. Plants were harvested after 30 days and the *Rhizobia* from 14–38 nodules per replicate were genotyped.

Results

None of the uninoculated control plants developed any nodules. *Rhizobia i*solated from nodules of plants inoculated with a single strain showed the expected antibiotic resistance.

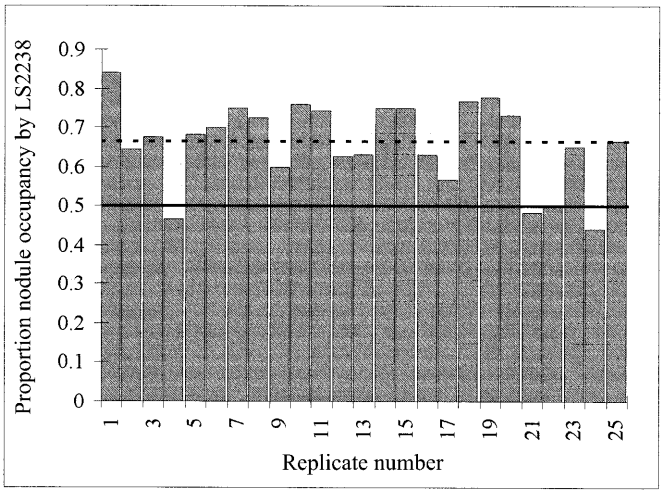

Fig. 1 Mean nodule occupancy by LS2238 after inoculation with a 1:1 LS2238: LS2202 mixture. The expected mean is shown by the solid line, the actual overall mean by the broken line

The experiment was designed to test the hypothesis that the parent and transgenic strain are equally able to nodulate the host when present together in the soil. Hence, we expect to see an average of 50% of nodules occupied by each strain after inoculation with a 1:1 mixture of the strains. From Fig. 1 it is evident that the mean nodule occupancy by LS2238, after co-inoculation, is higher than expected (mean=66.62%). There is variation between replicates, but in 21 of the 25 replicates the transgenic strain occupies a greater proportion of nodules than expected. From Table 1 it can be seen that the variation from a 1:1 ratio is significant, i.e. the transgenic forms significantly more nodules, on average, than the parent strain. It can also be seen that the variation between replicates is not significant at the 5% level. It is therefore concluded that this variation is no more than that expected due to chance.

Comparison between soil and axenic culture

The percentage nodule occupancy was converted into the number of LS2238 observed for every one expected if

Table 1 Chi-square analysis to test for variation between replicates, and from a 1:1 nodule occupancy ratio

Source of variation	χ^2	*df*	*P*	Inference
Total ($\Sigma\chi^2$)	108.53	24		
From 1:1 ratio	76.11	1	<0.001	Significant variation from a 1:1 ratio
Between replicates	32.42	23	>0.05	No significant difference between replicates

Table 2 Analysis of variance of the number of LS2238 observed for every one expected, showing a significant difference in nodule occupancy between plants grown in soil and axenic culture

Source of variation	Total	*df*	Mean	*F*	*P*
Total sum of squares about the mean	8.75	73			
Sum of squares between culture medium	2.65	1	2.65	33.13	<0.01
Residual sum of squares	6.10	72	0.08		

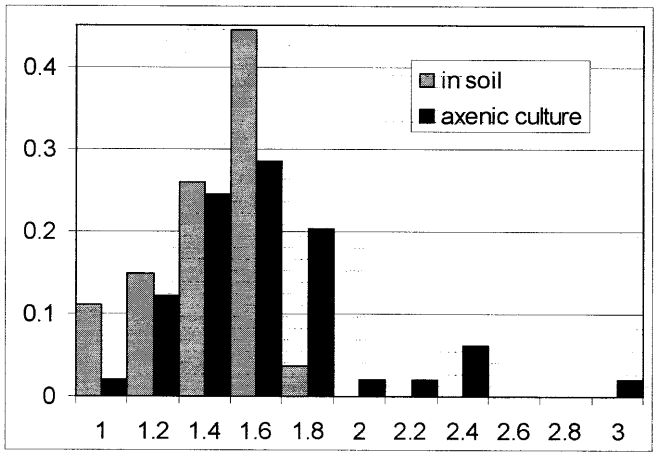

Fig. 2 Frequency distribution of the number of LS2238 observed for every one expected if there is no difference in the ability to compete for nodule sites. The upper limit of each category is shown on the x-axis (e.g. 1.8 means from >1.6 up to, and including, 1.8 nodules occupied by LS2238 per one expected)

there is no difference in the ability to compete for nodule sites. For the described experiment the overall mean and standard deviation are 1.32 and 0.22 respectively. Data taken from a directly comparable experiment with plants grown in axenic culture gave an overall mean and standard deviation of 1.47 and 0.38 respectively (49 replicates). Figure 2 shows the frequency distribution for this value in soil and axenic culture. There is some indication that the enhanced ability of LS2238 to compete for nodule sites is greater in axenic culture than in soil. Analysis of variance (Table 2) shows that this difference is significant.

Summary of results

(1) There were no significant differences between replicates.
(2) The increased ability to compete for nodule sites, previously observed in axenic culture, was shown to also occur in a soil environment.
(3) There was a significant difference in ability to compete for nodule sites in the different growing media; the enhanced ability was more pronounced in axenic culture.

Discussion

The observed secondary effect of transformation might be due to the transgenic construct causing an insertion mutation, particularly as transgenes are often inserted into the genome at random. This is probably made more likely by the fact that most of the genome of micro-organisms, such as *Rhizobia*, is of coding and related sequences, compared with eukaryotic genomes which contain substantial amounts of non-coding ”junk” DNA. Alternatively there may be pleiotropic effects of the transgene itself. Pleiotropy is common in bacterial mutants selected for novel phenotypes and is usually maladaptive, leading to reduced fitness (Lenski 1988a). Such adverse effects are, however, often ameliorated by selection for epistatic modifiers (Lenski 1988b). It is often assumed that any secondary effects of transformation will also cause reductions in fitness, although more recent evidence indicates that some transgenic oilseed rape exhibits pleiotropic effects that may positively affect fitness in some environments (Linder and Schmitt 1995). We plan to test whether the observed change in the ability to compete for nodule sites is due to pleiotropic effects of the inserted transgenic construct and, if it is, which part of the construct is responsible for the effect.

It seems unlikely that we picked, by chance, the only strain of the only organism in which there is a secondary effect of transformation. This being so, it is possible that other traits of other transgenics might be unexpectedly altered by transformation. Such characters might have a bearing on the safety and/ or success of those transgenics when they are released into the environment. Of particular concern are traits that affect fitness or dispersal. In the case of the *Rhizobia* the enhanced ability to compete for nodule sites might be a desirable feature of an inoculum. Strains with this character may be more-likely to associate with the crop and less-likely to form free-living colonies in 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. Nevertheless, the affect of an increased ability to compete for nodule sites on the fitness of *Rhizobia* is unclear, and future experiments are being planned to investigate this. In addition to the variation in this ability that is described here between different culture media there is preliminary evidence for other genotype by environment interaction for this character. This includes variation with temperature and, more pronounced, with host plant genotype. This last in particular will be an additional focus of future studies. The observed variation between culture media is probably due to there being additional inter-specific microbe interactions occurring in soil that are absent in the sterile conditions of axenic culture.

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