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Identification of genetic factors controlling the efficiency of *Agrobacterium rhizogenes*-mediated transformation in *Brassica oleracea* by QTL analysis

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Abstract We have identified quantitative trait loci (QTL) for transgenic and adventitious root production using an *Agrobacterium rhizogenes*-mediated co-transformation system in conjunction with a *Brassica oleracea* double haploid (DH) mapping population. Three QTL for green fluorescent protein (GFP)-fluorescent root production and four QTL for adventitious root production were identified as accounting for 26% and 32% of the genetic variation in the population, respectively. Two of the QTL regions identified were common to both transgenic and adventitious root production. Two different methods of QTL analysis were employed (marker regression and interval mapping) and with the exception of one region on linkage group O7 for transgenic root production, both techniques detected the same regions of the genome. The regions we identified to be associated with the control of transgenic root production following *A. rhizogenes*-mediated transformation are the first to be detected using a QTL mapping approach. In addition, this is the first study to identify genetic regions that co-regulate both transgenic and adventitious root production within the constraints of an *A. rhizogenes*-mediated transformation process. We have identified plant genotypes that do not produce any transgenic roots that may be deficient for T-DNA integration via illegitimate recombination and that may also be potentially important for the development of homologous recombination protocols. Conversely, we have also identified plant genotypes with high rates of transgenic root production that will be critical in the development of high throughput transformation systems.

Keywords QTL analysis · *Brassica oleracea* · Plant transformation · *Agrobacterium rhizogenes* · Green fluorescent protein

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Introduction

Agrobacterium-mediated transformation is the most widely used method to deliver transgenes into *Brassica* species (reviewed by Poulsen 1996; Puddephat et al. 1996). Efficient transformation into a wide range of genotypes is limited by genetic constraints imposed either by the plant or bacterial genome. A great deal of effort has been put into characterising the bacterial genes controlling T-DNA transfer (reviewed by Gelvin 2000) in an attempt to improve transformation efficiency by the manipulation of these factors, as recently applied in broccoli transformation by Henzi et al. (2000). By comparison, there have been few reports of plant genes that regulate the transformation process, despite strong evidence for a plant genotypic basis to transformation. It has been proposed that there are at least four steps in *Agrobacterium*-mediated transformation at which plant and bacterial genes could regulate the process: (1) bacterial attachment to the plant cell surface; (2) transfer of the T-DNA from the bacterium to the plant cell; (3) transport of the T-DNA to the plant nucleus; (4) stable integration of the T-DNA into the host genome (Gelvin 2000).

The transfer and integration of *Agrobacterium* T-DNA into the plant genome is mediated through expression of the *vir* genes present on the virulence plasmid native to *Agrobacterium* strains (reviewed by Kado 1991). The virulence genes are highly conserved, and the key events in T-DNA transfer are common to the two principal species of *Agrobacterium* used in plant transformation. Consequently, the analysis of plant genes regulating transfor-

mation using either *A. rhizogenes* or *A. tumefaciens* will allow an understanding of a process common to both.

Recent reviews have highlighted the variation in transformation responses between different *Brassica* species (Poulsen 1996) and, in particular, *B. oleracea* genotypes (Puddephat et al. 1996). There have been several reports of successful breeding for ease of transformation in *Lycopersicon esculentum* (Koornneef et al. 1987, 1993; Barg et al. 1997). *Solanum tuberosum* is genotype-dependent with respect to transformation efficiency and has a locus controlling *A. tumefaciens*-mediated transformation mapped to chromosome 5 (El-Kharbotly et al. 1995). *Arabidopsis thaliana* ecotypes have been shown to vary with respect to the proportion of explants producing transgenic calli (Schmidt and Willmitzer 1988; Sangwan et al. 1992). Other studies of *Arabidopsis thaliana* have shown heritable variation for *Agrobacterium tumefaciens* bacterial binding to the plant cell or reduced T-DNA integration (Nam et al. 1997). Further analysis of the plant genes involved has made use of T-DNA-tagged *A. thaliana* mutant lines (Nam et al. 1999). Mutant lines that are resistant to *A. tumefaciens* have been identified, and one line (*rat* 5), deficient in T-DNA integration, contains an inactivated histone H2A gene. Complementation studies of *rat* 5 show that histone H2A is important for T-DNA integration and that over-expression of the gene increases the proportion of transformation events in an *A. tumefaciens*-meditated transformation system (Mysore et al. 2000).

B. oleracea is an ideal crop species in which to study the genetic determinants of transformation due to its close relationship to *Arabidopsis thaliana* (Kianian and Quiros 1992; Kowalski et al. 1994; Sadowski et al. 1996; Truco et al. 1996) and the availability of homozygous double haploid (DH) mapping populations (Sebastian et al. 2000). Information developed in *B. oleracea* can be applied throughout the *Brassica* spp. as a result of the close relatedness of the species (U 1935). The DH mapping population documented by Bohuon et al. (1996) has already been utilised for the identification of quantitative trait loci (QTL) associated with flowering time (Rae et al. 1999), seed vigour and seedling growth traits (Bettey et al. 2000). The increased resolution of DH molecular marker maps, mapping populations and characterised substitution lines in *Brassica oleracea* (Bohuon et al. 1996; Sebastian et al. 2000) facilitate the identification of regions of the plant genome responsible for *Agrobacterium*-mediated plant transformation following QTL analysis.

In order to elucidate the genetic factors that regulate *Brassica* transformation by QTL analysis, experimental variation must be minimised. A standardised transformation system is therefore required. An optimised transformation system for *B. oleracea* has been developed utilising *A. rhizogenes* (Puddephat et al. 2001). We have already shown that the effects of plant genes regulating *A. rhizogenes*-mediated transformation of *B. oleracea* can be identified and established that plant genes controlling transformation segregate in a quantitative manner (Cogan et al. 2001). In addition, we have shown that there was no segregation distortion associated with transformation efficiency as a consequence of producing anther culture-derived DH lines from a range of *B. oleracea* germplasm.

A. rhizogenes has been shown to enhance adventitious root production during inoculation of *B. oleracea* (Puddephat et al. 2001). In addition, inoculation with *A. rhizogenes* has been shown to initiate both transgenic and non-transgenic adventitious roots in recalcitrant woody species (Lambert and Tepfer 1991; Yibrah et al. 1996; Rinallo et al. 1999; Falasca et al. 2000).

In the investigation reported here we aimed to assess the genetic variation that is present for *Agrobacterium*-mediated transformation. We present data from the analysis of a DH mapping population of *B. oleracea* for adventitious and transgenic root production. A total of seven QTL were identified for both adventitious and transgenic root production, with two QTL regions being identified as potentially common to both processes.

Materials and methods

Plant material and culture conditions

Initial transformation experiments were conducted using the parents of the *Brassica oleracea* AG mapping population (Bohuon et al. 1996; Sebastian et al. 2000), A12DHd (A) and GDDH33 (G) and the re-synthesised F_1 (A×G). Transformation experiments were performed on 73 lines of the AG DH mapping population (Bohuon et al. 1996; Sebastian et al. 2000). Seeds were surface-sterilised by immersion in a 1.7% (w/v) solution of sodium dichloroisocyanurate for 6 min, followed by two rinses in sterile purified water, then air-dried under aseptic conditions for at least 4 h. The dried seeds were transferred to 5-cm-deep petri dishes, which were subsequently sealed with Nescofilm prior to storage at 5 °C, in plastic bags containing silica gel.

Seeds were germinated in 25-compartment 100-mm-square repli dishes (Bibby Sterilin), with each compartment containing 1 ml of water-agar (6 g/l) and three seeds of each genotype. The dishes were incubated for 3 days at 15 °C under a 12/12-h (light/dark) photoperiod with light produced by an even mix of white and warm-white fluorescent tubes yielding an irradiance of 250 μmol m^{-2} s^{-1} at the culture level. They were then transferred for a further 3 days to 22 °C under a 16/8-h (light/dark) photoperiod with light provided by a mix of 70-W white and 65/80-W Gro-lux fluorescent tubes yielding an irradiance of 80 μmol m^{-2} s^{-1} at the culture level.

Transformation procedure

Agrobacterium rhizogenes strain LBA 9402 pRi1855 (Spano et al. 1982) was used, which harbours the binary plasmid pBIN-m-*gfp* 5-ER (Haseloff et al. 1997) bearing the green fluorescent protein (*gfp*) gene driven by a CaMV 35S promoter. Cultures were maintained on semi-solid YMB medium (Gelvin and Liu 1994) supplemented with 50 mg/l kanamycin for selection of the binary plasmid prior to transformation. From the overnight culture, three or four 10-μl loops of *Agrobacterium* were used to inoculate 10 ml of MGL broth (Walkerpeach and Velten 1994). These broth cultures were incubated for 16 h at 25 °C on a shaking platform (150–200 rpm). *Agrobacterium* cells were pelleted by centrifugation at 11,600 g for 5 min, and the cells resuspended in liquid Murashige and Skoog (1962) medium with 30 g/l sucrose (MS30) and supplemented with 1 mg/l 2-4-dichlorphenoxyacetic acid (2,4-D) to produce an optical density (A_{600nm}) of 1.0 (±0.1).

Explants for use in transformation experiments were excised from 6-day-old aseptic seedlings by cutting the hypocotyl approximately 5 mm below the cotyledons. The explants were inverted and placed on MS30 medium supplemented with 200 mg/l cefotaxime in 5 cm petri dishes with three explants of the same genotype per dish. A 3-µl drop of resuspended *Agrobacterium* was placed onto the cut surface of the hypocotyl. Inoculated explants were incubated at 22 °C under a 16/8-h (light/dark) photoperiod as described above.

Identification of co-transformed 'hairy roots'

Transformation events were detected by visual fluorescence of GFP in root tissues (referred to here as GFP-fluorescent roots). Inoculated explants were illuminated with long-wave UV using a hand-held lamp (UVP, B-100 AP). The number of GFP-fluorescing roots and the total number of roots produced by each explant were recorded.

Experimental design and data analyses

For the initial screen of the parental genotypes and re-synthesised F_1, *A. rhizogenes* inoculations were made to 48 explants (16 petri dishes, each containing 3 explants) of each genotype and the experiment repeated on five occasions. For the AG population screen, 16 replications of the following experimental design were performed: in each replicate, one petri dish for each of the 73 DH mapping lines plus the parents A12DHd and GDDH33 was produced; each petri dish contained three seedling explants which were inoculated with *Agrobacteria*.

The number of GFP-fluorescing and adventitious roots produced per explant were recorded 35 days after inoculation. Data were analysed using a generalised linear model (McCullaugh and Nelder 1989) with Poisson error and log-link function. Terms were fitted for replicate and genotype, and the estimated means for genotypes, averaged over replicates, were obtained for the QTL analysis.

QTL analyses

Genotype data were taken from the genetic linkage map described by Sebastian et al. (2000). A subset of marker loci was selected to provide even coverage of the genome with markers spaced approximately 10 cM apart. Consensus map distances were taken from Sebastian et al. (2000).

Mean data for GFP-fluorescent and adventitious root production were each analysed by ANOVA, per linkage group, by comparing the sum of the mean scores with marker position (marker-means). ANOVA at each marker was initially used to confirm that significant genetic variation was associated with markers on some chromosomes and to provide approximate locations for QTL. Subsequently, QTL analysis was performed by marker regression and interval mapping to explore these chromosomes in more detail using the QTL Café software (http:/web.bham.ac.uk/g.g.seaton/).

QTL analysis using marker regression was based upon the approach of Kearsey and Hyne (1994). QTL identification was performed by the use of ANOVA. A single predicted QTL was only accepted when the residual mean square figure was not significant at the 5% level and the regression mean square was significant at the 1% level. Where a single predicted QTL was significant using marker regression, the predicted location along with confidence intervals in centiMorgans and additive effects were calculated using 1,000 simulations. If the residual test statistic was significant, genetic models with additional QTL were examined. The interval mapping QTL analysis approach (Lander and Botstein 1989; Haley and Knott 1992; Jansen and Stam 1994) was also performed on the data set using the same software to confirm the presence of QTL. When the residual *F*-test statistic was significant but additional QTL models could not explain the data set, square root transformations were performed to improve the normality of the data. The improved normality will assess if the significant residual variation that could not be satisfactorily explained was an artefact of the distribution of the data set.

Results

Thirty-five days after inoculation of DH parental genotypes (A12DHd and GDDH33) and F_1 (A×G) explants, root production was observed from the cut surface of the majority of hypocotyls. Root formation was higher in explants of GDDH33 (99%) than in those of A12DHd (70%) but was approximately equal to the F_1 response (95%). The mean number of adventitious roots produced following inoculation with *A. rhizogenes* was 3.94 and 8.93 per explant for A12DHd and GDDH33, respectively (Table 1). The F_1 genotype produced a similar mean number of adventitious roots as GDDH33 (8.34). GFP was detected as a bright lime-green fluorescence in roots produced from a proportion of explants when cultures were illuminated with long-wave UV light. A12DHd and GDDH33 produced similar proportions of inoculated explants with GFP-fluorescing roots (14% and 20%, respectively). Mean GFP-fluorescent root production per explant was 0.39 and 0.66 for A12DHd and GDDH33, respectively (Table 1). In contrast, explants of the F_1 genotype had a much higher mean number (1.79) of GFP-fluorescent roots (Table 1).

Adventitious and transgenic root production following *A. rhizogenes*-mediated transformation was assessed in 73 DH lines and the parents of the AG mapping population. Of the 16 experimental replications, 13 generated useful data, and three were lost due to a growth room

Table 1 Generation means of GFP-fluorescent and adventitious root production for A12DHd (A) and GDDH33 (G), re-synthesised F_1 (A×G) and DH lines of the AG mapping population. Estimates of additive genetic effects and genetic dominance are derived from the generation means, where *m* is the mean, *a* is the estimate of additive effects and *d* is the effects of dominance

Genotype	Additive-dominance model	GFP fluorescent root production			Adventitious root production		
		$\bar{X}$ Mean	Estimate of *a*	Estimate of *d*	$\bar{X}$ Mean	Estimate of *a*	Estimate of *d*
A	*m* – *a*	0.39	0.14		3.94	2.50	
G	*m* + *a*	0.66			8.93		
A×G	*m* + *d*	1.79		1.26	8.34		1.91
AG mapping population	*m*	1.09			5.72		

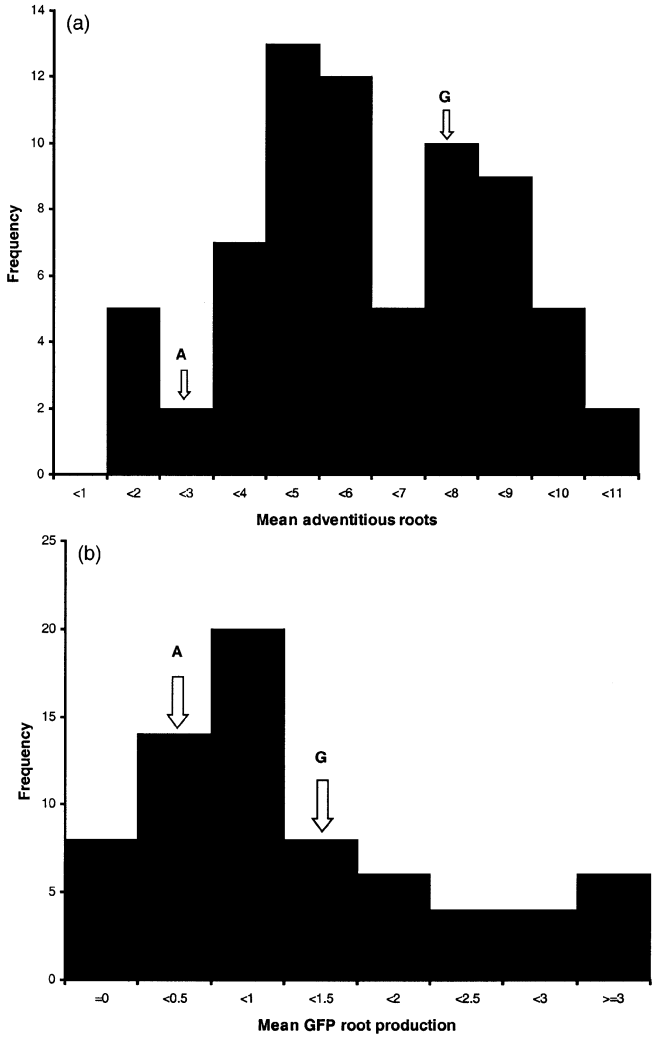

Fig. 1a, b Frequency histograms, mean scores of adventitious (**a**) and GFP-fluorescent root production (**b**) from seedling explants of the AG DH mapping population. Explants were scored 35 days after inoculation. *Arrows* indicate the mean score for adventitious and GFP-fluorescent root production for each parent: *A* A12DHd (2.44 and 0.3, respectively), *G* GDDH33 (7.55 and 0.65, respectively)

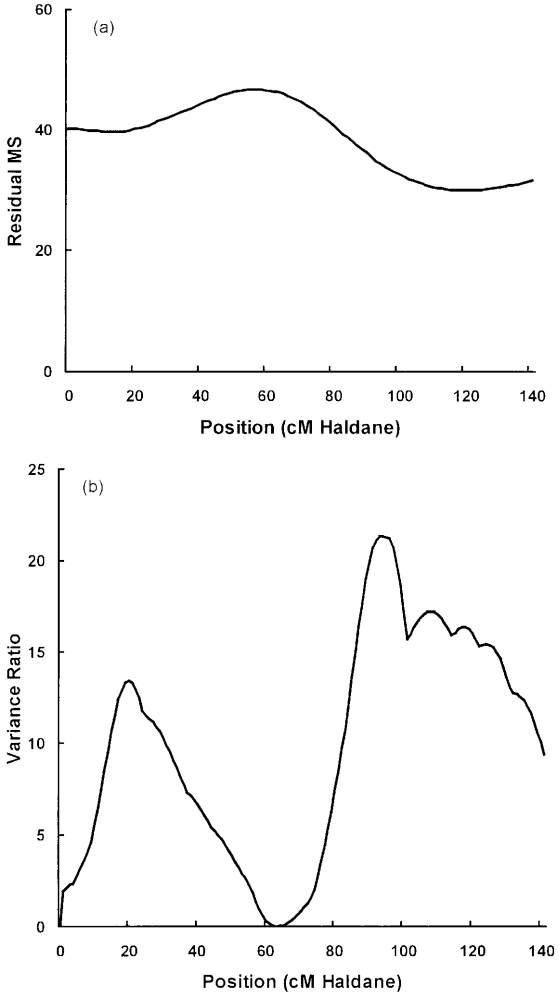

Fig. 2a, b Two QTL linked in repulsion on linkage group O3 of *Brassica oleracea* for adventitious root production. QTL were detected by marker regression (**a**) performing 1,000 simulations and interval mapping (**b**) using the QTL CAFÉ software available on http:/web.bham.ac.uk/g.g.seaton/. Genotype data for the mapping population were taken from Sebastian et al. (2000). Predicted QTL were only accepted when the residual mean square figure was not significant at the 5% level and the regression mean square was significant at the 1% level

failure. For each genotype, means for the performance of explants (transgenic and adventitious root production) were determined by analysis of deviance. The performance of the parental lines was consistent with the initial screen (Table 1, Fig. 1). For three AG DH lines, the means for adventitious and transgenic (GFP-fluorescent) root production were estimated with large standard errors (variances); in these lines either poor seed germination or explant contamination had reduced the number of explants screened; consequently, they were excluded from any further analysis of the data. The mean number of adventitious and GFP-fluorescent roots produced per explant segregated within the DH mapping population (Fig. 1a, b).

Highly significant differences ($P < 0.001$) were found between the lines for adventitious and GFP-fluorescent root production. Differences between the lines always produced the main treatment effect. Mean rates of adventitious rooting varied between repeats from 4.5 (±0.16) to 6.8 (±0.18) but accounted for significantly less variation (mean deviance = 12.9) than differences between the lines (mean deviance = 42.6). All DH lines produced adventitious roots, but rates of root production varied widely between lines from 1.4 (±0.4) to 10.8 (±1.2) per explant (Fig. 1a).

As with adventitious rooting, mean rates of GFP-fluorescent root production varied between the repeats from 0.7 (±0.06) to 1.6 (±0.09) but accounted for significantly less variation (mean deviance = 18.5) than differences between the lines (mean deviance = 33.9). Rates of GFP-fluorescent root production per explant varied widely between lines from 0±0 to 4.8±0.37. Eight DH lines failed

Table 2 Marker regression ANOVA statistics for all linkage groups of the AG mapping population screened for identification of QTL for GFP-fluorescent and adventitious root production (software – http://web.bham.ac.uk/g.g.seaton/). For adventitious root production on linkage group O3 the figures presented are for a two-QTL model. Values in bold-face type indicate significant additive regression for the acceptance of QTL

Linkage Group	GFP fluorescent root production				Adventitious root production			
	Additive regression		Residual		Additive regression		Residual	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
O1	756.25	**0.001**	56.97	**0.003**	3.91	0.493	1.37	0.043
O2	11.76	0.213	1.05	0.107	0.37	0.959	0.11	0.899
O3	632.98	**0.002**	18.37	**0.012**	245.86	**0.000**	1.32	0.226
O4	1.71	0.748	1.27	0.040	14.06	0.175	0.97	0.198
O5	0.37	0.938	0.23	0.625	68.07	**0.010**	0.59	0.413
O6	5.91	0.337	1.19	0.044	0.50	0.853	1.97	0.013
O7	47.57	0.036	0.27	0.766	302.85	**0.003**	4.30	0.070
O8	1.69	0.710	0.19	0.656	8.44	0.314	0.72	0.149
O9	17.24	0.156	3.57	0.020	10.17	0.302	0.70	0.273

to produce GFP-fluorescent roots in response to *A. rhizogenes* inoculation, while six DH lines produced more than 3.0 GFP-fluorescent roots per explant (Fig. 1b). The overall mean for GFP-fluorescent root production per explant in the AG DH population was 1.09 (Table 1).

Adventitious root production

As a preliminary analysis, the mean trait scores at each marker position were calculated and analysed by ANOVA (marker-means). The marker-means analysis identified two discrete regions on linkage group O3 (maximally $F = 14.13$ and 21.19, $P < 0.001$ for both) and regions on linkage groups O5 and O7 ($F = 15.86$, $P < 0.001$ and $F = 18.89$, $P < 0.001$, respectively) for adventitious root production. Significant genetic variation for adventitious root production was not associated with markers on any other linkage group.

For adventitious root production, there were significant regression scores using marker regression analysis on linkage groups O3, O5 and O7 ($P < 0.01$). There was no evidence for the presence of QTL on any other linkage groups (Table 2). For the QTL identified on linkage groups O5 and O7, the residual *F*-test statistic was not significant ($P > 0.05$), which indicates there was no evidence to support the presence of additional QTL on these linkage groups. When these data were analysed by interval mapping, the presence of a single QTL on linkage groups O5 ($F = 16.32$, $P < 0.001$) and O7 ($F = 18.84$, $P < 0.001$) was confirmed.

For linkage group O3, the single QTL analysis with marker regression was not significant (regression $F = 45.23$, $P = 0.079$; residual $F = 5.38$, $P = 0.062$). Preliminary marker-means analysis identified two regions as being significant. When a two-QTL model was examined the *F*-test statistic for regression was increased to $F = 245.86$, $P < 0.001$, and the residual *F*-test statistic was reduced to 1.32 ($P = 0.23$), indicating the presence of two linked QTL (Fig. 2a, b). The additive effects of these QTL (Table 3) indicate they are linked in repulsion. Interval mapping analysis confirmed the identification of two QTL on linkage group O3 linked in repulsion ($F = 19.84$, $P < 0.001$).

GFP-fluorescent root production

Marker-means analysis of GFP-fluorescent root production indicated that regions on linkage groups O1, O3 and O7 had a significant effect (LGO1 $F = 36.53$, $P < 0.001$; LGO3 $F = 11.756$, $P = 0.001$; LGO7 $F = 10.358$, $P = 0.002$) (data not shown). Subsequent marker regression analysis indicated that additive regression scores were significant ($P < 0.01$) for linkage groups O1 and O3, with the region on linkage group O7 approaching the threshold for acceptance of a QTL ($P = 0.036$). There was no evidence for the presence of QTL on any other linkage groups (Table 2). For the QTL identified on linkage groups O1 and O3, the residual *F*-test statistic was significant ($P < 0.05$), which may indicate the presence of additional regions of interest or QTL on these linkage groups. For linkage group O1, additional models for two QTL were tested, but further genetic regions could not be satisfactorily resolved. Interval mapping analyses were performed for linkage group O1, and a single region was identified conferring a significant ($F = 38.81$, $P < 0.001$) effect. This region corresponded with the single region identified by marker regression and initially detected by marker-means (data not shown). Square root transformation of the data was performed to improve normality. Subsequent analysis of the square root transformed data set by marker regression again identified a single region on linkage group O1 as being significant (regression $F = 48.95$, $P = 0.01$), with a non-significant residual ($F = 4.48$, $P = 0.113$).

For linkage group O3, additional models for two QTL were tested, but again genetic regions could not be satisfactorily resolved. Interval mapping analysis confirmed the presence of a single QTL at the same region

Table 3 Summary of QTL positions and effects for mean number of GFP-fluorescent and adventitious root production from the AG DH mapping population. Analysis was performed using the QTL CAFÉ software (http:/web.bham.ac.uk/g.g.seaton/) with confidence intervals calculated by Marker regression for position (centiMorgan) intervals and additive effects using 1,000 simulations. The QTL on linkage group O7 for GFP-fluorescent root production was only significant when interval mapping analysis was used; results from marker regression analysis are presented for comparative purposes

A: Marker regression

LG	Position	±	Effect	±	V_a	±%	h^2b
GFP-fluorescent root production							
1	100.37	14.10	0.58	0.180	8.84	0.86	0.09
3	85.36	22.56	0.45	0.186	5.37	0.92	0.05
7	61.71	17.80	–0.41	0.192	4.34	0.98	0.04
Total					14.21	1.78	
Adventitious root production							
3	34.00		–1.63				
	92.00		1.85		29.93		
5	38.70	11.22	–1.17	0.337	8.37	2.41	0.09
7	66.37	10.96	–1.09	0.349	7.80	2.50	0.08
Total					46.10		

B: Interval mapping

LG	Position	Effect	V_a	h^2b
GFP fluorescent root production				
1	105	0.75	15.00	0.14
3	88	0.49	6.46	0.06
7	72	–0.43	4.83	0.05
Total			26.29	
Adventitious root production				
3	27	–0.94	6.69	0.06
	90	1.25	8.96	0.10
5	38	–1.14	8.17	0.09
7	72	–1.11	7.91	0.08
Total			31.73	

(F = 10.73, P < 0.005). Square root-transformed data were analysed by marker regression, and residual scores were not significant (residual F = 0.95, P = 0.335); however, the regression score was above the threshold for reliable detection of QTL (regression F = 71.72, P = 0.036). Interval mapping analyses of the square root-transformed data set identified the same region as being significant (F = 18.33, P < 0.001).

For linkage group O7, marker-means analysis for GFP-fluorescing root production identified a single region as being significant. This region did not reach the threshold for significance when analysed by marker regression (P = 0.036), although by interval mapping analysis it was found to have a significant effect (F = 9.81, P < 0.005). Square root transformation of the data set did not improve the fit of the model by marker regression (regression F = 36.27, P = 0.054; residual F = 0.45, P = 0.527) or interval mapping (F = 11.33, P < 0.005).

The location and additive effect of each QTL identified from the analyses are summarised in Table 3. All of the predicted locations are consistent between the two types of analysis, with any variations being within confidence intervals. For example, the QTL on linkage group O1 for GFP-fluorescent root production was identified by marker regression at 100.37 cM, (±14.10 cM) and by interval mapping at 105 cM. The predicted effects of the individual QTL were also consistent between the two types of analysis with the greatest difference for GFP-fluorescent root production being on linkage group O1 (marker regression 0.58 ± 0.18, interval mapping 0.75); any variations are within the assigned confidence intervals. However, marker regression analysis for the two QTL for adventitious root production linked in repulsion on linkage group O3 does not produce confidence intervals associated with the position and effect; consequently, consistency between the two types of analyses can not be compared. The contribution of alleles from either parent in the AG population at each of the QTL identified for adventitious and GFP-fluorescent root production can be determined from the additive effect (Table 3). A positive-effect value indicates that the A12DHd allele enhances root production, while negative-effect values indicate that the A12DHd allele depresses the response. Consequently, transgenic root production was favoured by the presence of alleles from A12DHd on linkage groups O1 and O3 (positive-effect values, see Table 3) and alleles from GDDH33 on linkage group O7 (negative-effect values, see Table 3) at the respective QTL. Similarly, adventitious root production was favoured by the presence of A12DHd alleles on linkage group O3 (QTL at 90–92 cM) and GDDH33 alleles at the remaining three QTL (Table 3).

The estimate of the additive genetic effect at each QTL (V_a) compared to the total genetic effect observed in the population was calculated (Table 3). Using marker regression for GFP-fluorescent root production, a total of 14.2% of the total genetic variation observed in the pop-

ulation was explained by the QTL on linkage groups O1 and O3. For the same trait, interval mapping explains a total of 26.3% of the total genetic variation attributable by the three QTL identified on linkage groups O1, O3 and O7 and 21.46% of the total genetic variation by the QTL identified on linkage groups O1 an O3 alone. Analysis of adventitious rooting by marker regression accounts for 46.1% of the total genetic variation observed in the population. The V_a for the two QTL linked in repulsion on linkage group O3 was estimated by calculating the recombination fraction between the QTL. Adventitious root production analysed by interval mapping accounts for a total of 31.7%.

Broad-sense heritability is an indication of the level of effect each QTL has on the phenotype of the individual – in this case adventitious and transgenic root production. The broad-sense heritability values presented in Table 3 (indicated as h^2b), are equivalent to the V_a. In all cases, the results confirm the effectiveness of the experimental design used in this study.

Discussion

Transgenic root production segregated in the DH mapping population with variation behaving as a continuous distribution. This observation is consistent with our earlier findings that the process of anther or microspore culture to generate homozygous lines in *B. oleracea* does not distort segregation for transgenic root production in the resulting population (Cogan et al. 2001). Regions of the genome that contain enhancing QTL associated with transgenic root production originate from both parents, with the enhancing alleles coming from A12DHd for the QTL on linkage groups O1 and O3, while GDDH33 has an enhancing QTL allele on linkage group O7.

Initial QTL analysis using marker regression for transgenic root production indicated the possibility of additional QTL on linkage groups O1 and O3. Marker-means or additional QTL models could not resolve further significant regions. Furthermore, QTL analysis of the square root transformations of the data brings the residual *F*-test statistics above the threshold, indicating the presence of a single QTL on each linkage group. This suggests that the high residual mean squares were caused by variance or mean relationships. These three QTL explained up to 26.3% of the total genetic variation for transgenic root production, indicating that there are additional QTL that have yet to be identified, probably as their effects are individually too small to be detected in a population of this size.

Our results were based on scoring transgenic root production 35 days after inoculation and identified root structures that are known to give rise to stable transformed plants (Puddephat et al. 2001). Scoring at 35 days after inoculation identifies QTL associated with the production of a root expressing stably integrated transgenes. There are at least four stages in the process of *Agrobacterium*-mediated transformation that result in the stable integration and expression of transgenes (Gelvin 2000). It is possible that unidentified genetic elements control early events in the *Agrobacterium*-plant interaction. Scoring at an earlier stage may reveal QTL associated with bacterial binding or T-DNA transfer to the plant cell, whereas it is likely that the QTL we have identified here are important in T-DNA transfer and integration into the plant nuclear genome.

Four QTL were identified as being associated with adventitious root production. The parental means associated with adventitious root production show a much higher rate of root production originating from parent GDDH33 than from parent A12DHd (8.93 and 3.94, respectively). Of the four QTL identified, three have the enhancing allele originating from parent GDDH33 accounting for 22.8% of the genetic variation in the population.

The QTL identified on linkage groups O3 and O7 are common to both transgenic and adventitious root production. It is widely documented that the combined introduction of both the TL-and TR-DNA induces adventitious root production (Vilaine and Cassedelbart 1987; Capone et al. 1989; Zhan et al. 1990; Biondi et al. 1997). The induction of phenotypically normal non-transgenic adventitious root production upon *A. rhizogenes* infection has been previously reported in woody species and Chrysanthemum (van Wordragen et al. 1992; Yibrah et al. 1996; Falasca et al. 2000). In Chrysanthemum (van Wordragen et al. 1992), transient expression of the TR-DNA carrying the *iaa* genes was sufficient to induce adventitious root production by enhancing auxin levels. It was proposed that transient expression of the T-DNA regions could be sufficient to induce the proliferation of roots and callus, which could possibly indicate that the co-regulating regions we have identified control transient expression of the T-DNA complex. Consequently, the detection of loci that control both transgenic and adventitious root production is not surprising. The interaction between transgenic and adventitious rooting needs further investigation. This is the first study to identify genetic regions that co-regulate both transgenic and adventitious root production within the constraints of an *A. rhizogenes*-mediated transformation process.

The measurement of rates of root production, both transgenic and adventitious, for the parents and re-synthesised F_1 enables estimates of additive genetic effects and dominance to be calculated (0.14 and 1.26, respectively). An ‘A scaling’ test (Mather 1949) will determine if a simple ‘additive + dominance’ genetic model adequately explains the data set: there were no significant differences detected for transgenic or adventitious root production, indicating that the ‘additive + dominance’ genetic model adequately explains the variation (data not shown).

Gelvin (2000) has suggested that the generation of plants with a deficiency in T-DNA integration by illegitimate recombination may enhance our ability to effect homologous recombination in plants. Consequently, the identification of AG genotypes that are deficient for

transgenic root production could potentially be important for the development of homologous recombination protocols in *B. oleracea*. Conversely, the identification of plant genotypes with high rates of transgenic root production will be critical in the development of high-throughput transformation systems. The application of these QTL now enables us to identify recombinant genotypes in the AG mapping population that were not included in the transformation screen but in which transgenic root production is also highly efficient (data not shown). In the best performing lines identified in the population screen, up to 83% of inoculated explants produce transgenic roots. The best performing lines produce GFP-fluorescing roots from a far greater proportion of explants than the best performing parent, (20% for GDDH33 explants).

Henzi et al. (2000) used an *A. rhizogenes* transformation system for *B. oleracea*. By the manipulation of the inoculation protocol and by the addition of feeder cell layers (acetosyringone and opines), they were able to improve the transformation rate from 8% of the explants producing hairy roots to 33%. It has been proposed that the limit of transformation by the manipulation of *Agrobacterium* has almost been reached and that future advances will come from the manipulation of the plant host (Gelvin 2000). In this work we have used a genetic approach to identify genotypes which show an increase in transformation efficiency from 14% and 20% (parental transformation rates) to over 83%.

The main rate-limiting step in *Agrobacterium*-mediated transformation is the stable integration of the T-DNA into the host plant's nuclear genome (Gelvin 2000), which is regulated by plant proteins. Mysore et al. (2000) identified the Histone gene H2A, which controls T-DNA integration, by screening T-DNA tagged mutant lines of *Arabidopsis thaliana* with *Agrobacterium tumefaciens*. In contrast, we have identified the key genetic regions of the genome that regulate the trait. The regulation of transgenic root production is under complex genetic control. By analysing the trait in this way the identification of regions that regulate transgenic root production will be possible following more detailed analysis of the specific function of each QTL.

The identification and resolution of regions of the plant genome that control *Agrobacterium*-mediated transformation will assist in the development of genotype-independent transformation systems. Marker-assisted selection may be used to transfer favourable alleles to recalcitrant genotypes, thereby enabling the transformation of previously un-transformable germplasm. In addition, the identification of the genes underlying the QTL identified will ultimately provide a greater understanding of the processes occurring during T-DNA integration into the host plant genome. In order to identify the regulatory genes which may be present at the QTL, a specific function must be attributed to each region. And in order to assign function to the QTL, fine mapping, for example by the use of substitution lines for the *Brassica* DH population (Rae et al. 1999), will be required. The exploitation of colinearity between *B. oleracea* and *A. thaliana* by the identification of syntenous regions of the genomes that control the trait may accelerate the identification of key candidate genes.

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