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ORIGINAL PAPER

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Genetic analysis of *Agrobacterium tumefaciens* susceptibility in *Brassica oleracea*

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Abstract The genetic control and heritability of *Agrobacterium tumefaciens* susceptibility was investigated using a doubled haploid (DH) mapping population of *Brassica oleracea* and the associated RFLP map. Preliminary studies were carried out by analysis of an 8×8 diallel, for which the parental lines were selected to include a range of susceptibilities to *A. tumefaciens*. The variation observed within the diallel was attributed to both additive and dominant gene effects, with additive gene effects being more important. A broad sense heritability value of 0.95 suggested that 95% of the observed variation was due to genetic effects, with just 5% attributed to non-genetic or environmental effects. A high narrow-sense heritibility value of 0.79 suggested that 79% of this trait was controlled by additive gene effects and, therefore, the potential to introduce this trait into breeding material is high. Fifty-nine DH lines from the mapping population were screened for susceptibility towards *A. tumefaciens.* Variation in susceptibility was observed across the population. The results of the DH screen were entered into the mapping programme MAPQTL and a highly significant quantitative trait loci (QTL) associated with susceptibility to *A. tumefaciens* was identified on linkage group 09. The use of substitution lines covering this region confirmed the location of this QTL. This work shows that susceptibility to *A. tumefaciens* is a heritable trait, and the transfer of susceptibility into resistant lines is demonstrated. These findings may help to overcome genotype restrictions to genetic transformation.

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Introduction

Agrobacterium tumefaciens-based transformation systems rely on plants being susceptible to *Agrobacterium* infection; if it is not possible to introduce T-DNA into the plant cell, the selected genotype cannot be transformed. *Agrobacterium* is a natural plant pathogen that infects wounded dicotyledonous plants in vivo, and genetic variation for *A. tumefaciens* susceptibility has been observed in a wide range of plant species including *Prunus* (Bliss et al. 1999), soybean (Bailey et al. 1994; Mauro et al. 1995) and grape (*Vitis* spp.) (Szegedi and Kozma 1984). In these species, resistance to *Agrobacterium* is passed on to subsequent generations through both self- and cross-pollination, confirming the character to be a heritable trait. In grapevine, resistance to *A. tumefaciens* appears to be inherited as a single dominant gene, while in other species, such as soybean (Bailey et al. 1994; Mauro et al. 1995), susceptibility is dominant and often quantitative. In *Arabidopsis thaliana* (Nam et al. 1997) susceptibility again appears to be the dominant trait, and in one ecotype it may segregate as a single major contributing locus. Resistance to *Agrobacterium* in *Arabidopsis* has been associated with a deficiency in T-DNA integration (Nam et al. 1997, 1999) and several T-DNA-tagged mutants of *Arabidopsis* have been identified that are highly recalcitrant to *Agrobacterium* root transformation (*rat* mutants; *r*esistant to *Agrobacterium t*ransformation). Mysore et al. (2000) showed that in the *rat5* mutant, a histone H2A gene is disrupted. Complementation analysis and overexpression of *RAT5* indicates that H2A is an important factor in *Agrobacterium* transformation. The T-DNA integration stage of transformation is blocked in the *rat5* mutant. This suggests that H2A plays an important role in illegitimate recombination of T-DNA into the plant genome.

Agrobacterium-mediated transformation is the main method of choice for the genetic modification of plants. Considerable work has been done to improve bacterial strains, and it is now believed that future advances will come from the manipulation of the plants to be modified.

Over-expression of the H2A (*HTA1*) gene in wild-type *Arabidopsis* plants significantly increased susceptibility to *Agrobacterium*-mediated root transformation. The use of *HTA1* expression as a potential marker for predicting which cells are most susceptible to *Agrobacterium* infection is being explored (Gelvin 2003).

The aim of this study was to identify the inheritance patterns and genetic loci associated with susceptibility to *A. tumefaciens* in *Brassica oleracea*. In this investigation, susceptibility was not only an indicator of a genotype's ability to attract *A. tumefaciens* but also a measure of the uptake, integration and expression of the T-DNA. To facilitate visual screening, wild-type (tumour-producing) *A. tumefaciens* strains were used to test for susceptibility. The potential to introduce *A. tumefaciens* susceptibility into recalcitrant lines to alleviate genotype restrictions for genetic transformation is discussed

Materials and methods

Plant material

Fifty-nine doubled haploid (DH) lines from a reference mapping population, derived from a cross between *B. oleracea* ssp *alboglabra* (A12DHd) and *B. oleracea* ssp. *italica* (Green Duke GDDH33), were screened for susceptibility to *A. tumefaciens* (see experimental procedure). Five *B. oleracea* substitution lines (Ramsey et al. 1996) of the A12DHd genome with substituted regions of the GDDH33 genome covering linkage group 09 were supplied by Prof. M. Kearsey, Birmingham University. Inheritance patterns for susceptibility were studied using an 8×8 diallel cross. Eight DH lines from the mapping population were selected for diallel analysis based on their sensitivity to *A. tumefaciens* and included lines that were highly susceptible and lines that were more resistant to *A. tumefaciens.* The eight DH lines and the 56 F_1 families produced were screened for *A. tumefaciens* susceptibility.

Agrobacterium strains

Three wild-type strains of *A. tumefaciens* were used in this study: two nopaline strains (C58 and T37; Sciaky et al. 1978), and the octopine strain Ach5 (Ooms et al. 1981). *A. tumefaciens* strains C58 and T37 were obtained from N. McKenzie (John Innes Centre, Norwich, UK) and Ach5 from M. Christey (Crop and Food Research, Christchurch, New Zealand). C58 is the precursor to the disarmed strains AGL1, EHA 101 and EHA 105, and Ach5 the precursor to the disarmed strain LBA 4404. These disarmed strains are commonly used for transforming brassicas.

Experimental procedure

Seeds were surface-sterilised in 15% sodium hypochlorite for 15 min, rinsed in sterile water and germinated in Phytatrays (Sigma) containing MS salts , 3% sucrose and 0.8% Phytagar (Gibco BRL). Fine needles (Terumo 0.5×25 mm) dipped into a suspension of *A. tumefaciens* [$OD_{650\,nm}$=0.1] were used to infect 5-day-old seedlings just below the meristem. To ensure infection, 1.2 μl of the bacterial suspension was pipeted onto the injection site. Five seedlings were established per Phytatray containing MS medium (as above), making sure that the injection site was above the medium. Seedlings were transferred after 3 days to media (as above) supplemented with 500mgl^{-1} carbenicillin to eliminate *Agrobacterium* overgrowth. Five replicates for each of the 59 DH lines (with five explants per replicate) were established and inoculated with each of the three strains of *A. tumefaciens,* plus ten seedling controls (injected with Minimal A medium).

Thirty seedlings were inoculated with C58 for each of the eight DH parental lines and 56 F_1 lines of the diallel, and for the five substitution lines. Ten controls, injected with Minimal A medium, were included for each of the lines. All were screened after 50 days for the presence or absence of crown galls.

Statistical procedures

The proportion of seedlings forming crown galls was analysed using generalised linear models (Abeyasekera and Stern 2001) with logit link and binomial error using the software package Genstat version 5.0 (Genstat 1992). The integrated map was produced using JoinMap (Stam and Van Ooijen 1995). The data were entered into MAPQTL version 4.0 (Van Ooijen and Maliepaard 1996). Diallel analysis was performed using Genstat Version 5.0, according to Hayman (1954). Genetic component analysis was calculated according to Mather and Jinks (1971).

Results

Heritability of crown gall-formation

An 8×8 diallel analysis was carried out to investigate the heritability of *A. tumefaciens* susceptibility in *B. oleracea*. The diallel contained three DH parental lines that showed resistance to *A. tumefaciens* (DH 1027, 2069 and 5047) and five lines that showed high susceptibility to *A. tumefaciens* (DH 5117, 2072, 4030, 4052 and 6024) Table 1. The eight DH parental lines and the corresponding 56 F_1 lines were scored for the presence or absence of crown galls 50 days after infection with *A. tumefaciens* strain C58. The results are presented in Table 1 as mean frequencies (number explants with crown galls/total number explants infected). Crossing a DH line with low

Table 1 An 8×8 diallel table showing crown gall formation 50 days after inoculation with *Agrobacterium tumefaciens* C58. The data are presented as the mean frequencies (the number of explants forming crown galls/the total number of explants infected) from five replicates. Parental values are shown in *bold*

Female		Male							
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1	**DH 1027**	**0.04**	0.17	0.07	0.04	0.14	0.14	0.00	0.04
2	**DH 2069**	0.04	**0.10**	0.13	0.10	0.17	0.14	0.11	0.13
3	**DH 5047**	0.07	0.10	**0.17**	0.26	0.35	0.62	0.41	0.43
4	**DH 5117**	0.14	0.10	0.26	**0.87**	0.90	0.88	0.94	0.97
5	**DH 2072**	0.14	0.28	0.12	0.78	**0.72**	0.86	0.97	0.98
6	**DH 4030**	0.00	0.17	0.60	0.88	1.00	**0.90**	1.00	1.00
7	**DH 4052**	0.17	0.00	0.34	0.94	0.97	1.00	**0.94**	1.00
8	**DH 6024**	0.07	0.34	0.39	0.97	0.98	1.00	0.90	**1.00**

Table 2 Analysis of variance of an 8×8 diallel table for crown gall formation, after Hayman (1954). a Additive, b_1 measure of directional dominance, b_2 measure of ambidirectional dominance, b_3 measure of residual dominance, b dominance, c maternal effect, d reciprocal differences not ascribed to c, MS mean squares, *df* degrees of freedom, *F* variance ratio

Item	MS	*df*	*F*
a	2.2227	7	242.00***
b_1	0.2461	1	26.75***
b_2	0.0546	7	5.9***
b_3	0.1429	20	15.5***
b	0.1245	28	13.53***
c	0.0083	7	0.9 n.s.
d	0.0092	21	1.0 n.s.
Block error	0.0092	63	-

*** Significant at the 0.1% level; n.s. not significant

Table 3 Genetic components analysis, after Mather and Jinks (1971): D additive genetic variation, H_1, H_2 dominance genetic variances, F indicates relative frequency of dominant and recessive alleles in the parent population, E environmental effect

Component	Estimated value
D	0.16
H_1	0.12
H_2	0.11
F	–0.099
E	0.009
Mean degree of dominance $\sqrt{H_1/D}$	0.86
Proportion of dominance $H_2/4H_1$	0.22
Broad-sense heritability	0.95
Narrow-sense heritability	0.79

susceptibility (i.e. a crown gall frequency of less than 0.20) to another line with low susceptibility resulted in low crown gall-formation in the resulting F_1. Conversely, a high susceptibility×high susceptibility cross resulted in high gall formation in the resulting F_1 (a crown gall frequency of greater than 0.70). The frequency of crown gall formation in a low susceptibility×high susceptibility cross was generally much lower than the mid-point of the two parents, suggesting that low susceptibility (or resistance) to *A. tumefaciens* is partially dominant (Table 1).

No significant difference was observed between replicates of the diallel, but variation in crown gall formation was observed across the diallel. Two-way analysis of variance suggested that 5% of the variation, observed within the diallel table, was a result of non-genetic or environmental effects, and 95% of the variation was due to genetic effects. Comparing the mean of the DH parents (0.59) with the mean of the F_1 hybrids (0.46) suggested that low crown gall formation (or resistance to *Agrobacterium*) shows incomplete dominance over susceptibility to *Agrobacterium*. Further analysis of the diallel table, after Hayman (1954), revealed that both additive (a) and dominant (b) effects were significant for crown gall formation at the 0.1% level (Table 2), with additive effects being more important and accounting for the highest proportion of the total mean squares (MS). The majority of dominance (b) was accounted for by b_1, showing dominance to be unidirectional. Maternal effects (c, d) were not significant.

Genetic components analysis (Table 3) showed that additive variation (D) was larger than the dominance genetic variances (H_1 and H_2). The mean degree of dominance was 0.86, indicating incomplete dominance and supporting the comparison of the F_1 hybrid means. The broad sense heritability was 0.95 and this suggests that almost 95% of the variation observed in crown gall formation was due to genetic effects (with just 5% being due to environmental or non-heritable effects). The narrow sense heritability was also high at 0.79, and suggests that almost 79% of the genetic control was a result of additive gene effects.

Mapping QTL for crown gall formation

Fifty-nine DH lines from the mapping population were screened for susceptibility to three wild-type strains of *A. tumefaciens* (T37, C58 and Ach5). There was a significant difference in the frequency of crown galls produced between the two parental lines of the mapping population. GDDH33 was highly susceptible to *A. tumefaciens*, with nearly all seedlings (0.92) producing crown galls when inoculated, with each strain. In contrast A12DHd was much less susceptible and failed to produce crown galls when infected with the octopine strain Ach5 and the nopaline strain T37. Crown galls were observed when A12DHd was inoculated with C58 but the frequency was low (0.08; i.e. 2/25 explants produced galls). One-way analysis of variance showed no significant difference between replicates within lines, but a significant difference in crown gall formation was observed between DH lines across the population ($P<0.001$), showing susceptibility to *A. tumefaciens* to be genotype dependent. The distribution frequencies for crown gall formation across the DH population fell into similar low and high gall-forming classes as the two parents, Fig. 1. A proportion of the DH lines also showed an intermediate response to crown gall formation, so genotypes could be classified as showing low, intermediate or high crown gall formation when infected with T37 or C58.

Overall mean frequencies for crown gall formation across the DH population were Ach5 (0.38), T37 (0.61) and C58 (0.63) (Fig. 1). A high correlation of 0.92 ($P<0.01$) was observed between the frequencies of crown gall formation across the DH lines when inoculated with the two nopaline strains, T37 and C58.

The distribution frequencies for crown gall formation following inoculation with Ach5 showed less definition (i.e. did not fall into discrete groups) between the number of lines showing low and high crown gall formation. A higher proportion of DH lines failed to produce crown galls and fewer lines showed complete susceptibility to this strain of *A. tumefaciens.* This supports findings in the literature that octopine strains are less virulent than nopaline strains (Fry et al. 1987). Although the distribution frequencies observed for Ach5 were different, there

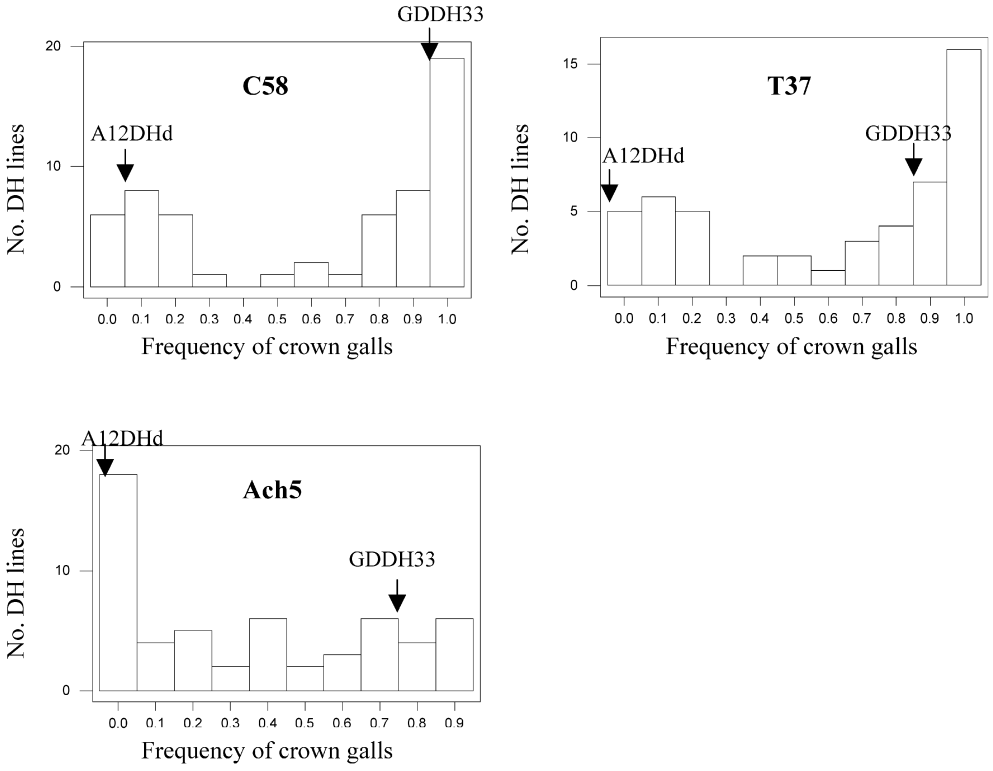

Fig. 1 Distribution in crown gall formation across the doubled haploid (DH) population, 50 days post-inoculation with C58, T37 and Ach5. The data are presented as frequencies (i.e. the number of explants forming crown galls/the total number of explants inoculated). The relative position of the two parental lines is indicated with *arrows*

was still a reasonable association between Ach5 and the two nopaline strains T37 and C58 (correlation coefficients of 0.75 and 0.78 respectively; $P<0.01$).

To identify the genetic loci involved in the control of susceptibility to *A. tumefaciens,* the frequency of crown gall formation for each of the three strains of *A. tumefaciens* were entered into MapQTL. The response of the DH lines to *A. tumefaciens* was then compared to the genetic map of this population. On linkage group O9 a large quantitative trait locus (QTL) was associated with susceptibility to each of the three *A. tumefaciens* strains, with highly significant LOD (logarithm of the odds) scores being observed (peaks of 9, 13 and 24 for Ach5, T37 and C58, respectively (Fig. 2).

Figure 2 illustrates that the two nopaline strains (T37 and C58) share the same QTL profile and that this profile is also the same as that associated with susceptibility to the octopine *A. tumefaciens* strain Ach5. Markers along the length of this linkage group are significantly associated with susceptibility to *Agrobacterium* with LOD scores of 3.0 or above covering most of linkage group O9. All three profiles peak at 40.1 cM and this position on the genetic map is associated with the RFLP marker pW233 (Sharpe et al. 1995).

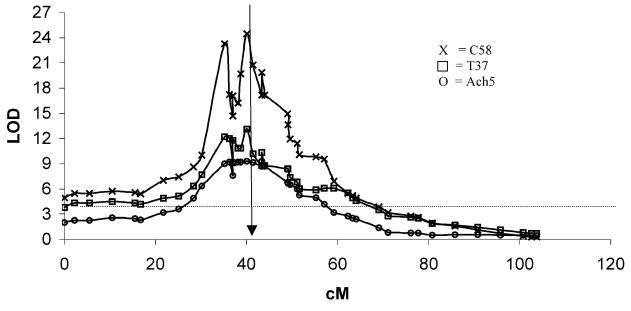

Fig. 2 Quantitative trait loci (QTL) profiles associated with *Agrobacterium tumefaciens* susceptibility, on linkage group O9. The QTL profiles associated with susceptibility to each of the *A. tumefaciens* strains had similar coverage across linkage group O9. Although the significance of each strain differed, all were above the level of significance, logarithm of the odds values ≥ 3.0 being significant (Van Ooijen 1999) as indicated by the *dotted line*. Each QTL profile hit a peak at 40.1 cM (*arrow*), and this represented the restriction fragment length polymorphism marker pW233

Confirmation of QTL on O9

Five substitution lines, produced using the same parental lines as the DH mapping population (Ramsey et al. 1996), were used to confirm the significance of this QTL. The basis of the substitution lines was the A12DHd genome into which varying lengths of the GDDH33 genome were intergressed. The five substitution lines contain fragments of the GDDH33 genome substituted into linkage group O9 covering the area to which the QTL associated with *A. tumefaciens* susceptibility has been mapped.

The five substitution lines and the two parental lines were inoculated with *A. tumefaciens* strain C58. A marked difference was again observed in the frequency of crown gall formation between the two parental lines. GDDH33 was highly susceptible to crown gall formation with a

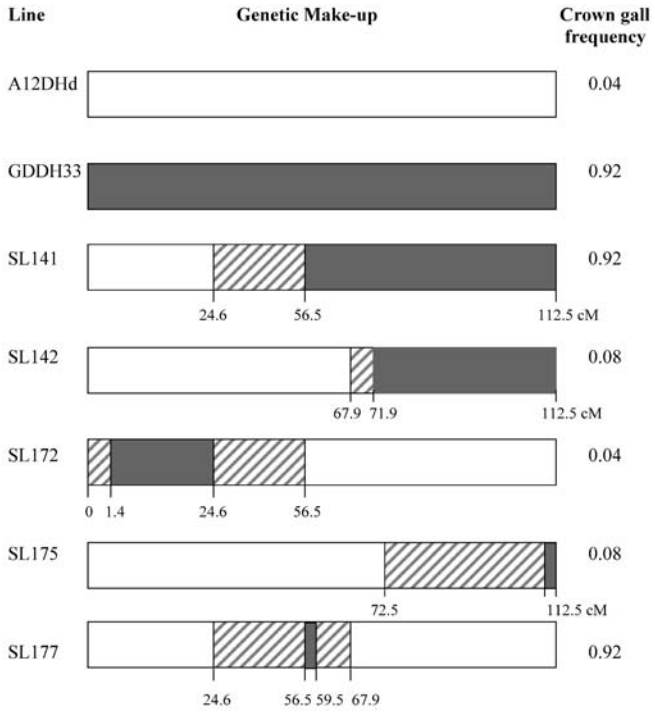

Fig. 3 Diagrammatic representation of five substitution lines (covering regions of linkage group 09) and the two parental genomes. The frequency of crown gall-formation 50 days post-inoculation with C58 is indicated. *Open bars* represent the A12DHd genome. *Solid bars* represent the GDDH33 genome. *Hatched bars* represent regions where recombination events have taken place, and therefore could be of either GDDH33 or A12DHd genetic background

frequency of 0.92, while A12DHd remained low with a frequency of 0.04. Substitution of the GDDH33 genome into the beginning or end of linkage group O9 (as in lines SL142, SL172 or SL175, Fig. 3), had no effect on susceptibility to *A. tumefaciens,* with gall production not significantly different to A12DHd. Introducing the GDDH33 genome into the central region of O9 (SL141 and SL177, Fig. 3) resulted in crown gall-formation at the same frequency as GDDH33 (0.92 for both substitution lines), and significantly different to A12DHd. This confirms that the central region of linkage group O9 plays an important role in the genetic control of susceptibility to *A. tumefaciens* and supports the identification of the QTL located in this region. The high-galling lines, SL141 and SL177, both contained GDDH33 sequences between 56.5 and 59.6 cM and it is possible that this region (which accounts for 2.7% of the genetic information of linkage group 09) is responsible for crown gall-formation. The map lengths associated with the substitution lines can not be directly compared to those of the DH map, however, since the two populations were generated at different times.

The regions where recombination events have taken place, however (indicated by the hatched areas in Fig. 3), can not be ignored because these areas could contain either of the two genomes. The region of linkage group O9 associated with *A. tumefaciens* susceptibility is therefore within the central region, between 24.6 and 67.9 cM, which accounts for 38.5% of the linkage group. The ability of substitution lines SL141 and SL177 to produce crown galls also demonstrates the ability to introduce genes associated with *A. tumefaciens* susceptibility, into otherwise recalcitrant lines.

The efficiency of *A. tumefaciens* as a transformation system will undoubtedly be affected if the genotype to be transformed is resistant to *A. tumefaciens*. Within this *B. oleracea* population, susceptibility to *A. tumefaciens* has been shown to be under genetic control, with some genotypes being susceptible and others resistant. The ability to select for susceptible lines may facilitate transformation, and the potential to use the RFLP probe pW233 (identified as that most closely associated with this trait) as a marker to predict susceptibility to *A. tumefaciens* was investigated.

Polymorphism associated with pW233 was noted for the two parental lines. DH lines that had previously been identified as high crown gall-forming lines gave the same banding pattern as GDDH33, while those lines identified as low crown gall formers had the same banding pattern as A12DHd (data not shown). Interestingly, those DH lines that produced crown galls at an intermediate level also shared the banding pattern of A12DHd. This suggests that lines with a degree of resistance to *A. tumefaciens* (observed by lines producing low or intermediate gall formation) share the banding of A12DHd, in this population. Substitution lines that contain the segment of the GDDH33 genome on linkage group O9, where the QTL associated with susceptibility to *A. tumefaciens* is located (SL141 and SL177), had the GDDH33 banding pattern. In contrast, SL142, SL172 and SL175 (low crown gall formers) had the A12DHd banding pattern. pW233 was successfully used to identify *B. oleracea* genotypes highly susceptible to *A. tumefaciens* and genotypes with low or intermediate susceptibility to *A. tumefaciens*, although it could not distinguish between the latter two (data not shown). Single marker analysis revealed that although pW233 was highly associated with crown gall formation, linkage was not complete, with 2 out of the 59 DH lines screened not showing a direct correlation (data not shown). As new microsatalite markers are assigned to this map (http://jic-bioinfo.bbsrc.ac.uk/BrassicaDB) it is hoped that future work will identify a more tightly linked marker that could potentially be used as a PCR-based screening method for *Agrobacterium* susceptibility in *B. oleracea*.

Discussion

Analysis of an 8×8 diallel showed that both additive and dominant gene effects control susceptibility to *A. tumefaciens*, with additive gene effects being more important. The high narrow-sense heritability value of 0.79 shows that around 79% of this trait is controlled by additive gene effects and, therefore, the potential to introduce this trait

into breeding material, through conventional breeding methods is high. The GDDH33 parental genotype (which showed a high frequency of crown gall formation) is derived from the commercial variety Green Duke. This would suggest that the introduction of susceptibility (or conversely, the removal of resistance genes) would not be detrimental to commercial varieties and, in fact, crown gall disease is not a commercial problem for *B. oleracea*.

A putative QTL associated with susceptibility to *A. tumefaciens* was located on linkage group O9 of the genetic map of *B. oleracea*. The use of substitution lines confirmed the presence of this QTL and suggests that genes responsible for susceptibility are located within the central 38%, of linkage group O9. Integration of the cytogenetic and genetic linkage maps of *B. oleracea* has recently been reported (Howell et al. 2002). This alignment of linkage groups with chromosomes suggests that genes associated with susceptibility to *A. tumefaciens* identified on linkage group O9 are located on chromosome 3 of the cytogenetic map of *B. oleracea.* Fine mapping of the gene or genes associated with susceptibility to *A. tumefaciens,* by further backcrossing of the key substitution line (SL 177) to A12DHd, could lead to the isolation and cloning of this gene(s)

The formation of crown galls following inoculation with wild-type *A. tumefaciens* enabled a quick visual reference for testing genotype susceptibility towards *A. tumefaciens*. In this investigation, susceptibility was not only an indicator of a genotype's ability to attract *A. tumefaciens* but also a measure of the uptake, integration and expression of the T-DNA. It should be noted, however, that crown gall formation and growth depend not only on the efficiency of gene transfer but also on the hormonal status of the plant (Puddephat et al. 1996). Further work by our group has demonstrated a strong correlation between crown gall formation in DH lines and β-glucuoronidase expression (r=0.81, P<0.01) when the same genotypes were transformed with disarmed strains of *A. tumefaciens* (data not shown), suggesting that the genetic control of crown gall formation, as described here, relates to susceptibility to *A. tumefaciens* and not to the hormonal status of the plant (i.e. the formation of the gall itself).

The A12DHd/GDDH33 population has also been screened for adventitious and transgenic root production following inoculation with *Agrobacterium rhizogenes* (Cogan et al. 2002). Three QTL were identified for adventitious root production on linkage groups O3, O5 and O7, and QTL associated with transgenic root production were assigned to linkage groups O1, O3 and O7. The QTL identified on linkage groups O3 and O7 were common to both adventitious and transgenic root production. The future screening of substitution lines in order to assign the precise function of these QTL was proposed. Further work on these QTL, and on the QTL identified in this paper will determine whether they are involved in specific aspects of infection such as, bacterial attachment, T-DNA transfer or T-DNA integration.

While the precise function of the gene(s) identified here are as yet unknown, the data presented in this current study clearly demonstrate that genetic loci associated with susceptibility to *A. tumefaciens* are located within the central region of linkage group O9 of *B.oleracea.*. The ability to select for, or introduce, genes that give rise to *A. tumefaciens* susceptibility will faciliate the use of *A.tumefacians* -based transformation methods for *B.oleracea*

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