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Identification of T-DNA tagged *Arabidopsis* mutants that are resistant to transformation by *Agrobacterium*

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Abstract We have identified T-DNA tagged *Arabidopsis* mutants that are resistant to transformation by *Agrobacterium tumefaciens* (*rat* mutants). These mutants are highly recalcitrant to the induction of both crown gall tumors and phosphinothricin-resistant calli. The results of transient GUS (β-glucuronidase) assays suggest that some of these mutants are blocked at an early step in the *Agrobacterium*-mediated transformation process, whereas others are blocked at a step subsequent to translocation of T-DNA into the nucleus. Attachment of *Agrobacterium* to roots of the mutants *rat1* and *rat3* was decreased under various incubation conditions. In most mutants, the transformation-deficient phenotype co-segregated with the kanamycin resistance encoded by the mutagenizing T-DNA. In crosses with susceptible wild-type plants, the resistance phenotype of many of these mutants segregated either as a semi-dominant or dominant trait.

Key words *Agrobacterium* · *Arabidopsis* · Plant transformation · T-DNA

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Introduction

Agrobacterium-mediated transformation is the method most commonly used for the genetic transformation of plant cells. The molecular events that occur in the bacterial cell prior to T-DNA transfer are reasonably well understood, and include induction of the virulence (*vir*) genes (Winans 1992), processing of the T-DNA from the Ti-plasmid (Stachel et al. 1986; Filichkin and Gelvin 1993), and the formation of bacterial channels for exporting the T-DNA (Ward et al. 1988; Thompson et al. 1988; Kuldau et al. 1990), possibly as a DNA-protein complex (the T-complex; Howard and Citovsky 1990). However, despite the wide application of *Agrobacterium* to the genetic transformation of plants, we do not understand many details of the unique and complex interactions that occur between the bacteria and plant cells. In particular, we know little about plant host factors involved in crown gall tumorigenesis. There are at least four steps in which plant factors are likely to be involved during *Agrobacterium*-mediated transformation: bacterial attachment to the plant cell surface, transfer of T-strands from bacteria to plant cells across the plant cell wall and membrane, transport of the T-complex to the plant nucleus, and stable integration of T-DNA into the plant genome (Zupan and Zambryski 1995, 1997; Sheng and Citovsky 1996). Plant proteins involved in these infection-related processes are probably also involved in basic cell processes such as wall biosynthesis, protein trafficking to the nucleus, and DNA repair and recombination.

We previously identified naturally occurring variation in susceptibility to *Agrobacterium* infection among a number of *Arabidopsis thaliana* ecotypes, and defined the basic mechanisms underlying this variation in some of these ecotypes (Nam et al. 1997). For example, the ecotype UE-1 is deficient in the T-DNA integration process, and ecotypes Bl-1 and Petergof are deficient in bacterial attachment to the plant cell. Although it is possible to isolate genes involved in these processes

using a positional cloning approach, we chose a different methodology for the identification and isolation of these and similar genes involved in transformation.

To identify plant genes involved in *Agrobacterium* infection, we screened T-DNA insertion mutant lines of *Arabidopsis* for recalcitrance to transformation following bacterial infection. The large degree of variation in transformation among ecotypes suggested to us that there would be many plant genes involved in this process, and therefore screening of these mutagenized lines would be fruitful. T-DNA (or transposon) tagging has a number of advantages over chemical or radiation mutagenesis in terms of the ease of recovery of the mutant (and subsequently the wild-type) alleles. By employing T-DNA vectors specifically designed for insertional mutagenesis, it is often possible to isolate regions of plant DNA flanking the insertion site by plasmid rescue or PCR-based cloning approaches (Feldmann and Marks 1987; Feldmann, 1991; Liu et al. 1995; Krysan et al. 1996; Frey et al. 1998; Mathur et al. 1998). Several mutant genes have been identified and/or isolated using these systems. These include an auxin responsiveness gene (Bennett et al. 1996), embryonic lethal genes (Errampalli et al. 1991; Franzmann et al. 1995), protein kinase genes (Krysan et al. 1996), and actin genes (McKinney et al. 1995). However, in many instances the mutant phenotype did not co-segregate with the genetic marker encoded by the T-DNA (Errampalli et al. 1991; Feldmann 1991; Van Lijsebettens et al. 1991; Koncz et al. 1992; Franzmann et al. 1995; Azpiroz-Leehan and Feldmann 1997). In this paper, we describe the isolation of several *Arabidopsis* mutants that are resistant or highly recalcitrant to transformation by *Agrobacterium*. A preliminary screen of one T-DNA insertion library (Feldmann and Marks 1987; Feldmann 1991) indicated that such mutants represent approximately 0.7% of the mutants in this collection. Somewhat surprisingly, many mutants showed a dominant or semi-dominant phenotype when crossed to wild-type plants. The mutants are blocked at a number of different points in the transformation process, as determined by the ability to express GUS activity transiently after infection by an *Agrobacterium* strain containing a T-DNA with an intron-containing *gusA* gene. Some mutant plants that expressed little or no transient GUS activity were found to be deficient in the ability to bind *Agrobacterium* to their roots.

Materials and methods

Growth of *A. thaliana*

Seeds of T-DNA insertion *Arabidopsis* mutants were obtained from the Arabidopsis Biological Resource Center at Ohio State University (Columbus, Ohio) We surface-sterilized seeds with a solution composed of 50% commercial bleach and 0.1% SDS for 10 min, then rinsed them several times with sterile distilled water. Seeds were placed on Gamborg's B5 medium (Gibco-BRL, Gaithersberg, Md.) containing kanamycin (50 μg/ml) and solidified with 0.75% Bactoagar (Difco, Detroit, Mich.). After keeping the plates at 4° C for 2 days, we incubated them for 7 days under a 16 h light/8 h dark regime at 25° C. We then transferred kanamycin-resistant seedlings individually into baby food jars containing solidified B5 medium without kanamycin, and grew them for 7–10 days to obtain roots.

Growth of *A. tumefaciens*

All *Agrobacterium* strains were cultured in YEP medium (Lichtenstein and Draper 1986) supplemented with the appropriate antibiotics (rifampicin, 10 μg/ml; kanamycin, 25 μg/ml) at 30° C. We washed overnight bacterial cultures with 0.9% NaCl and resuspended them in 0.9% NaCl at 2×10^9 cfu/ml for in vitro root inoculation.

In vitro root inoculation and transformation assays

We excised roots grown on the agar surface, cut them into small segments (approximately 0.5 cm) in a small amount of water, and blotted the root segments on sterile filter paper to remove excess water. The blotted bundles of root segments were transferred to MS basal medium [4.32 g/l MS minimal salts (Gibco-BRL), 0.5 g/l MES pH 5.7, 1 ml/l vitamin stock solution (0.5 mg/ml nicotinic acid, 0.5 mg/ml pyridoxine, and 0.5 mg/ml thiamine-HCl), 100 mg/l *myo*-inositol, 10 g/l sucrose, and 0.75% bactoagar] and 2–3 drops of the bacterial solution were placed on them. After 10 min, we removed most of the bacterial solution and cocultivated the bacteria and root segments at 25° C for 2 days.

For transient transformation assays, we infected the root segments with *A. tumefaciens* GV3101 (Koncz and Schell 1986) containing the binary vector pBISN1 (Narasimhulu et al. 1996). After 2 days of cocultivation, we rinsed the roots with water, blotted them on filter paper, and stained them with X-gluc staining solution (50 mM NaH_2PO_4, 10 mM sodium·EDTA, 300 mM mannitol, and 2 mM X-gluc, pH 7.0) for 1 day at 37° C. Roots were examined using a 2× magnifying lens. For quantitative measurements of GUS activity, we ground the roots in a microfuge tube containing GUS extraction buffer (50 mM Na_2HPO_4, 5 mM dithiothreitol, 1 mM sodium·EDTA, 0.1% Sarkosyl, 0.1% Triton X-100, pH 7.0) and measured GUS specific activity according to Jefferson et al. (1987).

To quantitate tumorigenesis, we infected root segments with wild-type *A. tumefaciens* A208. After 2 days, we rubbed the roots on the agar surface to remove excess bacteria, then washed the roots with sterile water containing timentin (100 μg/ml) to kill extracellular bacteria. Small root bundles (5–10 root segments) were transferred onto MS basal medium lacking hormones but containing timentin, and the plates were incubated for 4 weeks at 25° C.

For transformation of root segments to phosphinothricin (ppt) resistance, we inoculated root segments with *A. tumefaciens* GV3101 containing pCAS1. pCAS1 is a modified pGPTV-BAR binary vector (Becker et al. 1992) with a *nos-bar* gene as a selectable marker and a reporter gene β-glucuronidase (*uidA*) driven by a mannopine synthase promoter plus an octopine synthase activator. After 2 days, we transferred small root bundles onto callus-inducing medium (CIM is 4.32 g/l MS minimal salts, 0.5 g/l MES pH 5.7, 1 ml/l vitamin stock, 100 mg/l *myo*-inositol, 20 g/l glucose, 0.5 mg/l 2,4-dichlorophenoxyacetic acid, 0.3 mg/ml kinetin, 5 mg/l indole acetic acid, and 0.75% bactoagar) containing timentin (100 μg/ml) and ppt (10 μg/ml). We scored ppt-resistant calli after 4 weeks of incubation at 25° C.

Agrobacterium attachment assays

Arabidopsis seeds were germinated aseptically and seedlings were grown on solidified MS medium. Root segments were cut from plants about 7 days after germination and washed briefly in water. About 5–10 segments were then suspended in 2 ml of liquid (either water or 0.4% sucrose) and 20 μl of *A. tumefaciens* C58 (previously grown in Luria broth; Maniatis et al. 1982) was added. After 24 h,

we used a needle to pick up the root segments and transferred them to a drop of water for observation with a Zeiss Photoscope 2 using Nomarski optics.

Results

Identification of *Arabidopsis* mutants that are resistant to transformation by *Agrobacterium*

To screen for *Arabidopsis* mutant plants that showed an altered tumor formation response after infection with *Agrobacterium*, we used an in vitro root bundle inoculation assay. We inoculated small segments of surface-grown roots of individual 3-week-old T-DNA insertion-mutagenized T4 plants (Feldmann and Marks 1987; Feldmann 1991) with *A. tumefaciens* A208. This bacterial strain induces formation of large, green teratomas on *Arabidopsis* ecotype Ws, the parental ecotype used for mutagenesis (Nam et al. 1997). We placed the remaining shoot of each plant into solidified culture medium to allow root regeneration. After observing the results of the root inoculation, we transferred the re-rooted plants that showed a resistance response into soil and allowed them to set seeds for the recovery of progeny. Of approximately 3000 kanamycin-resistant plants screened, 21 plants (0.7%) were repeatedly found to be highly resistant to *Agrobacterium* infection. The selfed progeny of these mutants were tested for homozygosity in subsequent generations. Homozygous mutant plants were retested at least twice more to ensure that they had retained the *Agrobacterium* resistance phenotype. We termed these mutants *rat*, for resistant to *Agrobacterium* transformation. In addition, we identified 15 mutants that were weakly resistant to infection. However, we did not pursue characterization of this latter class of mutants. Figure 1 shows the results of infection of the wild-type parent ecotype Ws and two mutants, *rat3* (Fig. 1A) and *rat4* (Fig. 1C). These mutants developed only a few tumors that were significantly smaller than those induced on wild-type plants. Table 1 shows the results of tumorigenesis assays on all 21 mu-

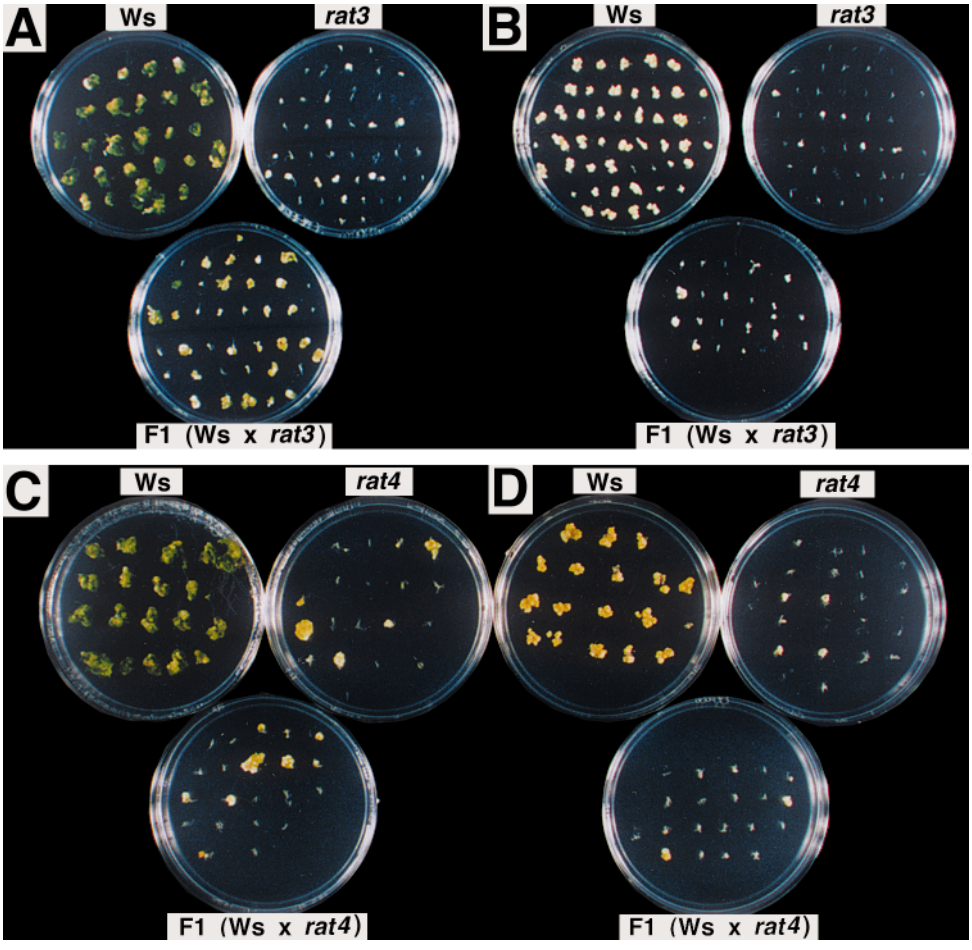

Fig. 1A–D Stable transformation of the *Arabidopsis rat3* and *rat4* mutants, the wild-type progenitor Ws, and their F1 progeny. Sterile root segments were infected with *A. tumefaciens* A208 (**A, C**) or GV3101(pCAS1) (**B, D**). After 2 days of cocultivation, the roots were moved to MS medium lacking phytohormones and containing timentin (**A, C**) or to CIM containing phosphinothricin and timentin (**B, D**). Tumors or phosphinothricin-resistant calli were scored after 4 weeks. **A** Crown gall tumorigenesis on roots of wild-type, *rat3*, and their F1 progeny. **B** Phosphinothricin-resistant calli formed on roots of wild-type, *rat3*, and F1 progeny. **C** Crown gall tumorigenesis on roots of wild-type, *rat4*, and F1 progeny. **D** Phosphinothricin-resistant calli formed on roots of wild-type, *rat4*, and F1 progeny

Table 1 Stable transformation of wild-type and *rat* mutant *Arabidopsis* plants by *Agrobacterium tumefaciens*

Line[a]	Percentage of root bundles with tumors	Tumor morphology	Percentage of root bundles with ppt-resistant calli[b]
Ws	86 ± 15	Large, green teratomas	87 ± 10
rat1	7 ± 1	Very small, yellow	5 ± 2
rat3	10 ± 4	Very small, yellow	9 ± 2
rat4	19 ± 8	Very small, yellow	14 ± 4
rat5	8 ± 3	Very small, yellow	15 ± 5
rat6	16 ± 5	Very small, yellow	12 ± 10
rat7	18 ± 10	Very small, yellow	10 ± 5
rat8	36 ± 5	Large and small, yellow	40 ± 5
rat9	4 ± 5	Very small, yellow	6 ± 4
rat10	3 ± 4	Very small, yellow	19 ± 5
rat11	4 ± 5	Very small, yellow	14 ± 3
rat12	5 ± 6	Very small, yellow	10 ± 5
rat13	5 ± 8	Very small, yellow	20 ± 10
rat14	6 ± 5	Very small, yellow	9 ± 5
rat15	13 ± 5	Medium, yellow	11 ± 6
rat16	21 ± 10	Small, green	33 ± 11
rat17	18 ± 12	Very small, yellow	20 ± 12
rat18	21 ± 7	Small, yellow	27 ± 8
rat19	4 ± 3	Very small, yellow	9 ± 4
rat20	10 ± 6	Very small, yellow	31 ± 10
rat21	8 ± 9	Very small, yellow	21 ± 8
rat22	17 ± 8	Small, yellow	29 ± 15

[a] At least five different plants were tested for each mutant and 40–50 root bundles were tested for each plant
[b] ppt-resistant calli produced by all mutants were slightly smaller than those produced by the wild-type plant

tants. We scored tumorigenesis as the percentage of infected root bundles that produced any type of callus or teratoma. However, the relatively high percentage of tumorigenesis shown by several mutants generally reflected the generation of small, yellow calli. In no case did these mutants produce large, green teratomas similar to those generated on wild-type Ws roots.

To determine whether these *rat* mutant lines were deficient in their ability to be transformed to other stable phenotypes, we infected them with the nontumorigenic strain *A. tumefaciens* GV3101 containing the binary vector pCAS1. pCAS1 contains a chimeric *nos-bar* gene. We used ppt resistance as a stable selectable marker for transformation, because these *rat* mutants were already transformed with a T-DNA containing a kanamycin resistance (*nptII*) gene. The results of these experiments, shown in Table 1, indicated that all *rat* mutants were recalcitrant to transformation to ppt resistance. Although in some mutants a higher percentage of root segments developed ppt-resistant calli than developed tumors, these calli tended to be very small compared to those generated on the roots of the wild-type ecotype Ws. Figure 1 shows the ppt resistance phenotype of the wild-type ecotype Ws and the mutants *rat3* (Fig. 1B) and *rat4* (Fig. 1D).

Preliminary characterization of the *rat* mutants

We have conducted a preliminary characterization of the *rat* mutants in an attempt to determine at what point *Agrobacterium*-mediated transformation is blocked. All plants appeared phenotypically normal when germinated. Root segments from all mutants tested formed calli on callus-inducing medium without selection. These calli grew with the same frequency and rate as did those of wild-type Ws plants (data not shown). These data suggest that the *rat* mutants tested to date do not have any deficiencies in cell division or cell proliferation.

To determine which steps in the *Agrobacterium*-mediated transformation process were disrupted in the *rat* mutants, we compared the efficiency of T-DNA transfer to, and expression in, these mutant plants with that of the wild-type plant by quantitatively measuring both transient GUS (β-glucuronidase) activity and comparing this to the efficiency of stable transformation (incidence of tumorigenesis and ppt resistance). We determined the relative transient transformation efficiency by inoculating sterile root segments with *A. tumefaciens* GV3101 harboring the T-DNA binary vector pBISN1, and staining for GUS activity using the chromogenic dye X-gluc. pBISN1 contains an intron-containing *gusA* gene under the control of a "super-promoter" (Ni et al. 1995; Narasimhulu et al. 1996). Because of the intron in the *gusA* gene, GUS staining represents activity directed by the T-DNA after transfer to the plant rather than activity resulting from leaky expression of the *gusA* gene in *Agrobacterium* (Liu et al. 1992). Using this vector, we could detect GUS activity after cocultivation for only 2 days. This early expression of GUS activity most probably represents transient expression of genes harbored by the T-DNA that has not yet integrated into the plant genome (Nam et al. 1997). Table 2 shows the results of these experiments. Almost all root segments (92%) of the wild-type ecotype Ws stained blue with X-gluc, indicating that this ecotype is highly susceptible to

Table 2 Transient GUS expression in Ws and *rat* mutants

Plant	Percentage of root bundles stained blue with X-gluc[a]
Ws	92 ± 6
rat1	22 ± 4
rat3	31 ± 2
rat4	10 ± 4*
rat5	86 ± 2
rat6	9 ± 2
rat7	18 ± 10*
rat8	30 ± 8
rat9	20 ± 4*
rat10	26 ± 12
rat11	24 ± 4
rat12	19 ± 5
rat13	11 ± 2*
rat14	13 ± 1*
rat15	18 ± 2
rat16	28 ± 7
rat17	90 ± 6
rat18	88 ± 5
rat19	18 ± 3
rat20	83 ± 10
rat21	20 ± 8
rat22	70 ± 5

[a] At least three different plants were tested for each mutant and at least 100 root segments were observed for each plant. *Asterisks* indicate that the spots were very small and stained light blue

Agrobacterium-mediated transformation. Most of the *rat* mutants showed significantly reduced levels of transient transformation, as reflected by the relatively low percentage of root segments that showed only a few small, light-blue spots after staining with X-gluc. However, five mutants (*rat5*, *rat17*, *rat18*, *rat20*, and *rat22*) showed levels of staining that were approximately as high as in the wild-type plants. Because these five mutants were difficult to transform to the stable phenotypes of tumorigenesis and ppt resistance, our results suggest that they are blocked at the step of T-DNA integration into the plant genome.

Genetic analysis of *rat* mutants

To determine the genetic characteristics of the *rat* mutants, we backcrossed several of the homozygous mutant plants, using the mutant (kanamycin resistant) as the pollen donor, to wild-type (ecotype Ws; kanamycin sensitive) plants and selected F1 hybrid plants by germinating seeds of each cross on B5 medium containing kanamycin. Most F1 hybrid plants displayed either an intermediate response in the tumorigenesis and ppt resistance tests (semi-dominant phenotype; *rat1*, *rat3*, and *rat8*) or a dominant mutant phenotype (*rat4*, *rat5*, *rat6*, *rat7*, *rat9, rat10, rat11, rat12,* and *rat13*). *rat 22* is the only recessive mutant obtained to date. Figure 1 shows the results of these transformation experiments with the semi-dominant mutant *rat3* (Fig. 1A and B) and the dominant mutant *rat4* (Fig. 1C and D). In the F2 populations, the kanamycin resistance phenotype segregated 3:1 (Kan^r:Kan^s) as a dominant characteristic for all tested mutants except *rat10*, indicating that a single linkage group was disrupted by T-DNA insertions in these mutants. However, the numbers of T-DNAs integrated in each mutant line could be different. Kanamycin resistance segregated 15:1 in the F2 population that resulted from crossing *rat10* to the wild-type plant, indicating that this mutant line contains two independently segregating, expressed *nptII* genes.

To examine the co-segregation of the T-DNA insertion with the *rat* phenotype, we grew individual F2 plants from selected crosses on solidified B5 medium without kanamycin. We cut roots from individual plants, infected half the bundles of root segments with *A. tumefaciens* A208 and transferred them onto MS basal medium without hormones to induce tumors. We transferred the remaining half of the root bundles onto callus-inducing medium containing kanamycin to screen for kanamycin-sensitive (k/k) calli. Finally, we transferred the remaining plant shoot first to B5 medium without kanamycin to induce root formation, then into soil to obtain F3 seeds. For those lines showing a semi-dominant phenotype, we germinated the seeds on B5 medium containing kanamycin to determine the genotype of the kanamycin-resistant plants among the F2 progeny. Table 3 shows that for the semi-dominant mutants *rat1*, *rat3*, and *rat8*, the genotypes kanamycin resistance and tumorigenesis each segregated as 1:2:1 [(k/k:K/k:K/K) and (susceptible:intermediate:resistant)]. If these mutants were semi-dominant, all F2 plants that are homozygous (k/k), heterozygous (K/k), and homozygous (K/K) for the kanamycin resistance gene should show susceptible, intermediate, and resistant phenotypes for tumorigenesis, respectively. We found that F2 plants sensitive to kanamycin (k/k) were uniformly as suscep-

Table 3 Co-segregation analysis of semi-dominant *rat* mutants

Mutant	Number of plants tested	Phenotype[a]			χ^2 value[b]
		Kan^r/Tum^-	Kan^r/Tum^I	Kan^s/Tum^+	
rat1	44	10	20	14	1.09*
rat3	50	7	35	8	8.04
rat8	46	15	23	8	2.13*

[a] Tum–, tumorigenesis deficiency; Tum^I, intermediate phenotype; Tum^+, tumorigenesis
[b] Test for 1:2:1 segregation of tumorigenesis. An *asterisk* indicates that the value is not significantly different from that expected at $P = 0.05$

tible to tumorigenesis as the wild-type parent. However, F2 plants resistant to kanamycin (K/k and K/K) did not always segregate with the expected phenotype. Some heterozygous plants (K/k) showed a fully resistant tumorigenesis phenotype similar to that of homozygous plants (K/K). In contrast, some homozygous plants (K/K) showed an intermediate tumorigenesis phenotype similar to that of heterozygous plants (K/k). This confusing result may derive from the fact that it is difficult to distinguish between the intermediate and resistant tumorigenesis phenotypes. However, the co-segregation of kanamycin sensitivity (k/k) with the susceptible phenotype, and the lack of susceptible plants that are homozygous for kanamycin resistance indicate that the *rat* mutant phenotype is tightly linked to the locus into which the T-DNA has integrated.

Table 4 shows that for five dominant *rat* mutants tested, the kanamycin-resistant and tumorigenesis-deficient phenotypes completely co-segregated. Thus, for these mutants the *rat* mutant phenotype is tightly linked to the locus into which the T-DNAs integrated. For one mutant not shown in Table 4 (*rat11*) the tumorigenesis-deficient phenotype did not co-segregate with kanamycin resistance, although the T-DNA tag was linked to the locus of interest.

rat1 and *rat3* are deficient in *Agrobacterium* attachment to roots

We initially chose the mutants *rat1* and *rat3* for more detailed characterization. Figure 2A shows that, when assayed quantitatively for GUS activity using the fluorescent substrate 4-methylumbelliferyl β-D-galactoside (MUG), these mutants displayed approximately 25% transient GUS activity compared to wild-type plants. These data correlate well with the data presented in Table 2. In the stable transformation analyses, *rat1* and *rat3* showed approximately 10% tumor formation and 10% induction of ppt-resistant calli, respectively, compared to the wild-type plants (Fig. 2B and C). Thus, *rat1* and *rat3* most probably have mutations that block tumorigenesis at an early stage in the transformation process. We therefore determined whether these mutants are blocked in the bacterial attachment step of transformation.

To examine the attachment of *Agrobacterium* to the *rat1* and *rat3* mutants, we incubated sterile root segments from these mutant plants with *A. tumefaciens* C58. We conducted attachment assays either in water or in 0.4% sucrose. Figure 3 shows that the wild-type plant Ws bound bacteria both in water (Fig. 3A) and in sucrose (Fig. 3D). The *rat1* mutant was highly deficient in its ability to bind *Agrobacterium*, both in water (Fig. 3B) and in sucrose (Fig. 3E). The *rat3* mutant was also unable to bind *Agrobacterium* in water (Fig. 3C); however, attachment occurred in sucrose (Fig. 3F). Thus, *rat1* and *rat3* roots are defective in their ability to bind *A. tumefaciens*.

Discussion

We have identified and partially characterized 21 *Arabidopsis* mutants, designated *rat* mutants, that are resistant to transformation by *Agrobacterium*. In contrast to the large green tumors with teratomas induced upon roots of the wild-type progenitor (ecotype Ws) in response to inoculation with *A. tumefaciens* A208, these mutants were almost completely unable to form tumors. This deficiency in forming tumors was paralleled by a deficiency in transformation to phosphinothricin resistance when root segments were infected with a nontumorigenic *A. tumefaciens* strain containing a binary vector harboring a chimeric *nos-bar* gene. Thus, these mutants are not merely deficient in their response to the phytohormones whose synthesis is directed by the incoming T-DNA (Morris 1986).

Genetic analysis of these *rat* mutants revealed that in most lines, the T-DNA inserted at a single locus. For most mutants examined, the T-DNA (as defined by an active kanamycin resistance gene) co-segregated with the *rat* mutant phenotype. Therefore for these mutants, the T-DNA either "tags" the gene responsible for the *rat* phenotype or is genetically closely linked to the gene responsible for this phenotype. Co-segregation of the T-DNA tag with the mutant phenotype in such a high percentage of mutants is unusual; most investigators have reported that in the majority of mutant lines examined, the T-DNA does not co-segregate with the mutant phenotype (Errampalli et al. 1991; Feldmann 1991; Van Lijsebettens et al. 1991; Koncz et al. 1992;

Table 4 Co-segregation analysis of dominant *rat* mutants

Mutant	Number of plants tested	Phenotype		χ^2 value[a]
		Kan^r/Tum^-	Kan^s/Tum^+	
rat4	98	70	28	0.67*
rat5	102	77	25	0.01*
rat6	40	31	9	0.13*
rat7	51	39	12	0.06*
rat9	49	39	10	0.55*

[a] Test for 3:1 segregation of kanamycin resistance and tumorigenesis. An *asterisk* indicates that the value is not significantly different from that expected at $P = 0.05$

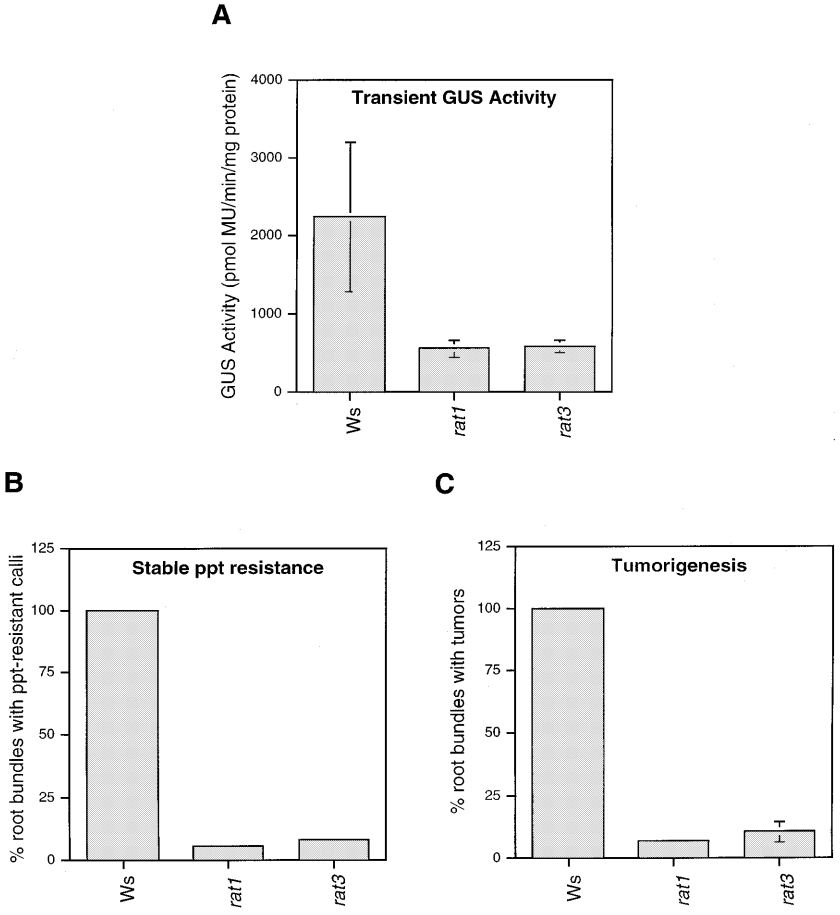

Fig. 2A–C Quantitative determination of the frequency of transient and stable transformation of the *Arabidopsis rat1* and *rat3* mutants and the wild-type progenitor Ws. **A** Sterile *Arabidopsis* root segments were infected with *A. tumefaciens* GV3101(pBISN1). After cocultivation for 2 days, transient GUS activity was determined using a quantitative, fluorimetric MUG assay. **B** Sterile *Arabidopsis* root segments were infected with *A. tumefaciens* GV3101(pCAS1). After 2 days of cocultivation, the root segments were transferred onto CIM containing 10 μg/ml phosphinothricin and timentin. The percentage of root bundles containing herbicide-resistant calli was scored after 4 weeks. **C** Sterile *Arabidopsis* root segments were infected with *A. tumefaciens* A208. After 2 days of cocultivation, tumors were induced on MS basal medium without hormones and containing timentin. The percentage of root bundles showing tumors was scored after 4 weeks. In **B** and **C**, the the frequency of ppt resistance and tumorigenesis for the wild-type Ws was reproducibly 100%. Where not indicated, the range of variation for the *rat1* and *rat3* mutants was too small to be depicted on the graph

Franzmann et al. 1995; Azpiroz-Leehan and Feldmann 1997). However, final confirmation that the T-DNA has disrupted the gene responsible for the *rat* phenotype can only come from genetic complementation of the mutant line with the wild-type gene corresponding to the locus into which the T-DNA has integrated.

Most of the *rat* mutants identified to date display the same phenotype. They are highly recalcitrant to transformation to several stable phenotypes, and they are also recalcitrant to transient transformation, as determined by GUS activity measured 2–3 days after infection. The difficulty experienced in transforming these mutants to both transient and stable phenotypes suggests that they are blocked at an early step in the *Agrobacterium*-mediated transformation process. This hypothesis is in accord with our finding that the *rat1* and *rat3* mutants are deficient in binding *Agrobacterium* to their roots. This lack of attachment in water suggests that there is a surface alteration in these plants. The finding that the *Rat1* gene encodes an arabinogalactan protein (Nam et al., in preparation) is consistent with this hypothesis. The effect of added sucrose probably operates on the plant because it has no effect on bacterial attachment to the *rat1* mutant. In addition, the presence or absence of sucrose has no effect on the binding of wild-type or attachment-deficient bacteria to wild-type plants (Fig. 3 and A. Matthysse, unpublished observations).

The *rat5*, *rat17*, *rat18*, *rat20*, and *rat22* mutants represent a particularly interesting subset of *rat* mutants. The phenotype of these mutants is characterized by high levels of transient transformation but low levels of stable transformation. Because even transient expression of the T-DNA-encoded intron-containing *gusA* gene requires T-DNA nuclear targeting and conversion of the single-stranded T-DNA to a double-stranded, transcription-proficient molecule (Narasimhulu et al. 1997), these mutants are likely to be deficient in T-DNA integration into the plant genome. Because T-DNA integrates into the plant chromosomes by a process of illegitimate re-

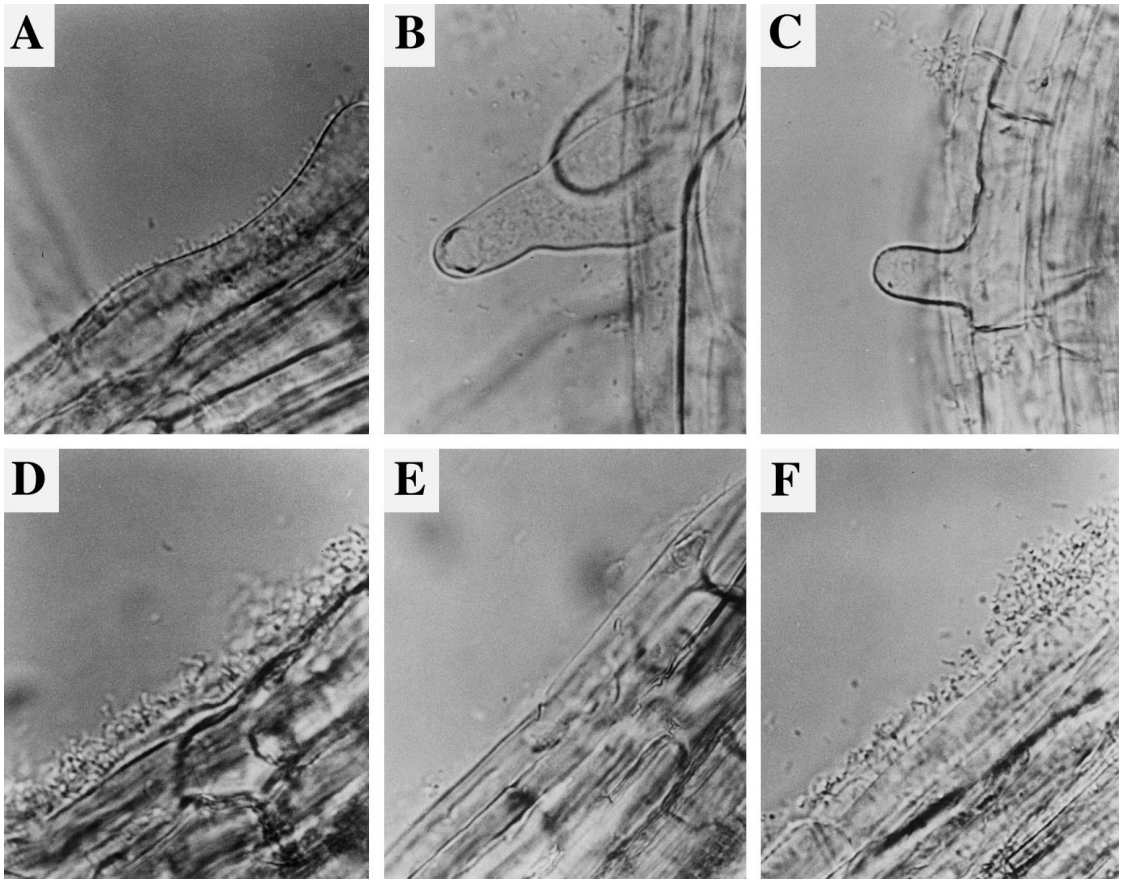

Fig. 3A–F Attachment of *A. tumefaciens* C58 to root segments of the *Arabidopsis* mutants *rat1* and *rat3* and their wild-type progenitor Ws. Root segments were incubated with bacteria for 24 h. in either water (**A–C**) or 0.4% sucrose (**D–F**). The roots were gently washed in water and observed under a microscope using Nomarski optics. **A** Wild-type roots in water. **B** *rat1* roots in water. **C** *rat3* roots in water. **D** Wild-type roots in sucrose. **E** *rat1* roots in sucrose. **F** *rat3* roots in sucrose

combination (Matsumoto et al. 1990; Gheysen et al. 1991; Mayerhofer et al. 1991; Ohba et al. 1995), this class of *rat* mutants may identify plant genes involved in DNA repair and recombination processes.

We were interested in determining whether the mutations in these *rat* mutants were allelic. Because of the dominant or semi-dominant nature of the mutations, we were unable to establish allelism by simple crosses between the mutants. We have, however, cloned T-DNA/plant DNA junctions from the *rat1*, *rat3*, *rat4*, and *rat5* mutants and shown by hybridization to recombinant inbred lines that the disrupted genes map to different regions of the *Arabidopsis* genome (Nam et al., in preparation). In addition, cosmid clones corresponding to the *rat7*, *rat9, rat 17*, and *rat 22* T-DNA insertion sites do not contain DNA homologous to the T-DNA insertion sites of the *rat1*, *rat3*, *rat4*, or *rat5* mutants. Thus, no two of the eight molecularly characterized *rat* mutants appear to have lesions in the same gene. Given the high percentage of T-DNA insertion mutants that are *rat* mutants (0.7% of the approximately 3000 mutant lines tested to date), and the number of genes encoded by *Arabidopsis* (20,000–30,000), it is likely that there will be on the order of 200–300 plant genes involved in the *rat* phenotype. Although this appears to be a large number of genes, it is not without precedent. Several laboratories have reported a high frequency of *Arabidopsis* mutants that are hypersensitive to radiation (Davies et al. 1994; Masson et al. 1997). The large number of plant genes involved in *Agrobacterium*-mediated transformation reflects the complexity of the *Agrobacterium* infection process and probably includes genes involved in bacterial binding to the plant wall, T-DNA transfer and nuclear targeting, and T-DNA integration into the plant genome.

F1 progeny derived from crosses between the susceptible wild-type parent and several *rat* mutants exhibited an intermediate transformation phenotype, indicating that *rat1, rat3*, and *rat8* are semi-dominant mutants. All other *rat* mutants tested to date, except *rat22*, are dominant. *rat22* is the only recessive mutant that has been obtained so far. These surprising results imply that the susceptibility of *Arabidopsis* to *Agrobacterium* infection is sensitive to the copy number of these genes. We hypothesize that the majority of plant genes involved in *Agrobacterium*-mediated transformation are vital to the survival of the plant. This may explain our ability to recover only a very low frequency of recessive *rat* mutants as most mutants in the homozygous state would be lethal to the plant. In each of the eight dominant or semi-dominant *rat* mutants from which we have

cloned T-DNA/plant DNA junctions, the T-DNA has inserted into 5′ or 3′ untranslated regions of the genes (data not shown; Nam et al., in preparation). Such insertions into non-coding regions of the genes may allow for "leaky" gene expression. Such low levels of expression may be sufficient for survival of the plant, but not adequate for *Agrobacterium* transformation. If this hypothesis were correct, then we should be able to complement even dominant mutations by overexpression of the wild-type gene in the mutant genetic background. Indeed, we have been able to complement homozygous *rat3* and *rat5* (which has a disrupted histone H2A gene) mutant plants by transformation with the corresponding wild-type genes (Nam et al.; Mysore et al., in preparation). Alternatively, the products of some *rat* genes in which the T-DNA/plant DNA junctions have not yet been obtained may function by formation of homo- or hetero-multimers. Thus, mutation of one gene may decrease the number of functional gene products (in the case of a homo-multimer) or produce a partially inactive complex (in the case of a hetero-multimer, a dominant negative mutation; Herskowitz 1987).

The identification and partial characterization of *Arabidopsis* mutants that are resistant to *Agrobacterium* transformation presented in this paper provide a starting point for a detailed study of the molecular and biochemical mechanisms that control the T-DNA-mediated transformation process. Isolation and analysis of *Arabidopsis* genes necessary for *Agrobacterium*-mediated transformation may allow us to develop new ways to transform agronomically important plants that are currently recalcitrant to *Agrobacterium*-mediated genetic transformation.

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