Research Note

Agrobacterium tumefaciens* Transformation of the Radiation Hypersensitive *Arabidopsis thaliana* Mutants *uvh1* and *rad5

Jaesung Nam,¹ Kirankumar S. Mysore,² and Stanton B. Gelvin^{1,2}

¹Department of Biological Sciences, and ²Purdue Genetics Program, Purdue University, West Lafayette, IN 47907-1392 U.S.A.

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The *Arabidopsis thaliana* mutants *uvh1* and *rad5*, originally identified as radiation hypersensitive, were reported to be deficient in T-DNA integration based on the relative efficiencies of stable transformation and T-DNA transfer. We reassessed these mutants for susceptibility to transformation by *Agrobacterium tumefaciens*. The mutant *rad5* showed a significant reduction in the efficiency of transient as well as stable transformation, compared with its wild-type progenitor. These data indicate that *rad5* is blocked at a step in the transformation process prior to T-DNA integration. We additionally found, using both an in vitro root inoculation and an in vivo flower bolt inoculation assay, that the mutant *uvh1* is as susceptible to *A. tumefaciens*-mediated transformation as is its wild-type progenitor, C10.

Additional keywords: crown gall tumors.

Stable transformation of plant cells by nonreplicating DNA molecules predominantly results from integration of exogenous DNAs into random rather than homologous sites of the plant genome (Paszowski et al. 1988; Offringa et al. 1990). There is evidence that genotoxic stress (DNA damage) may affect the integration of exogenous DNA into the plant genome. Irradiation of protoplasts treated with exogenous DNA yielded a higher transformation efficiency, compared with non-irradiated protoplasts transformed under the same conditions. These results suggest that a higher integration efficiency of exogenous DNA into the plant genome following radiation may result from the activation of the plant recombination and repair machinery (Köhler et al. 1989, 1990).

Generally, ionizing radiation (fast-neutron, X-ray, or γ -ray) produces double- and single-strand breakage of plant genomic DNA (McClintock 1984; Shirley et al. 1992). Organisms have a number of mechanisms to maintain genetic integrity following such DNA damage. For example, DNA repair systems recognize and repair damaged DNA, and cell cycle checkpoint systems arrest cell division to allow time for DNA repair (Carr and Hoekstra 1995; Lydall and Weinert 1996; Jackson 1996;

Britt 1996). These mechanisms are highly conserved among eukaryotic cells. However, our understanding of the molecular mechanisms of DNA repair and checkpoint control processes in plants is in its infancy. Recently, several *Arabidopsis thaliana* mutants hypersensitive to the growth-inhibitory effects of ionizing radiation were isolated (Harlow et al. 1994; Davies et al. 1994; Jenkins et al. 1995; Masson et al. 1997). Although it is not known which defective plant factors are responsible for hypersensitivity to ionizing radiation in these mutants, it is suspected that these mutants are defective in DNA repair or checkpoint control processes.

Because the T-DNA of *Agrobacterium tumefaciens* is integrated into the plant genome by illegitimate recombination (Matsumoto et al. 1990; Gheysen et al. 1991; Mayerhofer et al. 1991; Ohba et al. 1995), chromosome breaks may play a role as a target site for T-DNA integration (Puchta et al. 1996), and double-strand DNA breaks are preferentially repaired by nonhomologous recombination in plants (Shirley et al. 1992), it has been speculated that the integration of T-DNA into the plant genome may be affected by DNA repair systems.

Sonti et al. (1995) reported that two *A. thaliana* mutants, *uvh1* (UV-hypersensitive) and *rad5* (γ -radiation hypersensitive), could not be stably transformed by *A. tumefaciens* or *A. rhizogenes* to yield crown gall tumors or kanamycin-resistant calli. According to these authors, however, both of these mutant plants could be transformed to yield transient β -glucuronidase (GUS) expression. The authors therefore concluded that these *A. thaliana* mutants were deficient in T-DNA integration (Sonti et al. 1995).

Previously, we identified naturally occurring variation in susceptibility to *A. tumefaciens* infection among a number of *A. thaliana* ecotypes. We defined the basic mechanisms for variation in some *A. thaliana* ecotypes. For example, UE-1 is deficient in the T-DNA integration process, and ecotypes An-1, BI-1, and Petergof are deficient in bacterial attachment to the plant cell (Nam et al. 1997). Because the reported *A. tumefaciens* transformation resistance of the mutants *uvh1* and *rad5* and that of *A. thaliana* ecotype UE-1 appeared similar, we re-examined the transformation characteristics of *uvh1* and *rad5*. Surprisingly, we found that the *uvh1* mutant was as easily transformed to yield crown gall tumors as was its wild-type progenitor, C10 (Columbia background). For these initial

Corresponding author: Stanton B. Gelvin; Telephone: 1-765-494-4939; Fax: 1-765-496-1496; E-mail: gelvin@bilbo.bio.purdue.edu

Fig. 1. Stable transformation of *Arabidopsis thaliana* mutants *uvh1* and *rad5* and their wild-type progenitors, C10 and Col-0. **A**, Sterile root segments were infected with *Agrobacterium tumefaciens* A208. After 2 days of cocultivation, the root segments were transferred to Murashige and Skoog basal medium without hormones and containing timentin (100 mg/liter). Tumors were scored after 4 weeks. **B**, Sterile root segments were infected with *A. tumefaciens* GV3101(pBISN1). After 2 days of cocultivation, root segments were transferred to callus-inducing medium (CIM) containing kanamycin (50 mg/liter) and timentin (100 mg/liter). Antibiotic-resistant calli were scored after 4 weeks. **C**, Sterile root segments were infected with *A. tumefaciens* GV3101(pCAS1). After 2 days of cocultivation, root segments were transferred to CIM containing phosphinothricin (10 mg/liter) and timentin (100 mg/liter). Herbicide-resistant calli were scored after 4 weeks.

tests, we used *A. tumefaciens* A208, harboring the nopaline-type Ti-plasmid pTiT37, in both an in vitro root bundle inoculation assay (Fig. 1A; Nam et al. 1997) and a flower bolt inoculation assay (Fig. 2 and Table 1; Nam et al. 1997). These results were quantitated (Fig. 3C) and confirmed by repeating experiments with *uvh1* and wild-type progenitor seeds supplied by R. V. Sonti. In addition, seeds lots were sent to Corrine Davies (University of Arizona, Tucson) and confirmed as radiation-sensitive (Davies et al. 1994). *A. tumefaciens* A348 and A281, containing the octopine-type Ti-plasmid pTiA6 and the super-virulent Ti-plasmid pTiBo542, respectively, induced small tumors on both the *uvh1* mutant and C10 (Table 1). Because we had previously determined that extensive preculture of root segments on medium containing hormones prior to inoculation with *A. tumefaciens* could result in misleading tumorigenesis results (Nam et al. 1997), the explants we used for tumorigenesis assays were never exposed to hormones.

In contrast to the above data, almost no kanamycin-resistant calli were induced on root segments of the mutant *uvh1* with *A. tumefaciens* GV3101 harboring the binary T-DNA vector pBISN1 that contains a *nos-nptII* gene (Figs. 1B and 3B; Nam et al. 1997). However, the wild-type plant C10 also showed a severe defect in the induction of kanamycin-resistant calli on

infected root segments (Fig. 1B). The 80% kanamycin-resistance transformation efficiency of C10 depicted in Figure 3B would appear to indicate efficient stable transformation of this line. However, these calli grew very poorly on callus-inducing medium (CIM) containing kanamycin, as shown in Figure 1B. We thus suggest that the difference in tumorigenesis- and kanamycin-resistance phenotypes noted by Sonti et al. (1995) may have resulted from slower development of transformed tissue in the mutant than in the wild-type, especially with the octopine-type (LBA4404) *A. tumefaciens* and *A. rhizogenes* strains used by these authors.

Table 1. Flower bolt inoculation of *Arabidopsis thaliana* lines with various *Agrobacterium tumefaciens* strains

<i>A. tumefaciens</i> strain	<i>A. thaliana</i> line ^a			
	C10	<i>uvh1</i>	Col-0	<i>rad5</i>
A136 (no pTi)	– (1)	– (1)	– (1)	– (1)
A348 (pTiA6)	++ (5)	++ (5)	++ (5)	+/- (20)
A208 (pTiT37)	++++ (9)	++++ (19)	++++ (5)	++ (19)
A281 (pTiBo542)	++ (12)	++ (5)	++ (15)	+/- (6)

^a ++++ = large tumors completely covering wound sites; ++ = some small tumors around wound sites; +/- = very few small or no tumors around wound sites; – = no tumors; (n) = number of plants tested.

Fig. 2. Tumorigenesis of *Agrobacterium tumefaciens* on flower bolts of *Arabidopsis thaliana* mutants *uvh1* and *rad5* and their wild-type progenitors, C10 and Col-0. Flower bolts of *A. thaliana* were inoculated with *A. tumefaciens* A208 (2×10^{11} CFU/ml) and the plants photographed 4 weeks later.

To determine whether the mutant *uvh1* could be transformed to the stable phenotype of phosphinothricin (ppt) resistance, we inoculated roots of the mutant and wild-type progenitor C10 with *A. tumefaciens* GV3101(pCAS1). pCAS1 is derived from pGPTV-BAR (Becker et al. 1992) and contains a *nos-bar* gene as well as a "super-promoter" (Ni et al. 1995) directing the expression of a *gusA* gene. We incubated the root

segments on CIM containing phosphinothricin and scored ppt-resistant calli after 1 month. Figures 1C and 3D show that the mutant *uvh1* was as susceptible as C10 to transformation to ppt-resistance. Taken together, the tumorigenesis, kanamycin-resistance, and phosphinothricin-resistance data indicate that roots of *uvh1* are as susceptible to transformation to various stable phenotypes as are roots of its wild-type progenitor C10.

Fig. 3. Quantitative determination of transient and stable transformation frequencies of *Arabidopsis thaliana* mutants *uvh1* and *rad5* and their wild-type progenitors, C10 and Col-0. **A**, Sterile *A. thaliana* root segments were infected with *Agrobacterium tumefaciens* GV3101(pBISN1). After 2 days of cocultivation, transient β -glucuronidase (GUS) activity was determined with a quantitative methylumbelliferyl- β -glucuronide (MUG) fluorimetric assay. Variation in the number of GUS units for the mutant *rad5* (± 221 GUS units) was too small to indicate. **B**, Sterile *A. thaliana* root segments were infected with *A. tumefaciens* GV3101(pBISN1). After 2 days of cocultivation, root segments were transferred onto callus-inducing medium (CIM) containing kanamycin (50 mg/liter) and timentin (100 mg/liter). Percentage of root bundles containing kanamycin-resistant calli was scored after 4 weeks. **C**, Sterile *A. thaliana* root segments were infected with *A. tumefaciens* A208. After 2 days of cocultivation, tumors were induced on Murashige and Skoog basal medium without hormones and containing timentin (100 mg/liter). Percentage of root bundles showing tumors was scored after 4 weeks. Lines C10, *uvh1*, and Col-0 each consistently showed 100% of root bundles with tumors. **D**, Sterile *A. thaliana* root segments were infected with *A. tumefaciens* GV3101(pCAS1). After 2 days of cocultivation, root segments were transferred onto CIM containing timentin (100 mg/liter) and phosphinothricin (10 mg/liter). Percentage of root bundles containing phosphinothricin-resistant calli was scored after 4 weeks. Lines C10, *uvh1*, and Col-0 each consistently showed almost 100% of the root bundles with tumors.

We additionally tested the ability to transform *uvh1* to kanamycin-resistance with *A. tumefaciens* GV3101(pBISN1) and a flower vacuum-infiltration transformation assay (Bechtold et al. 1993). Table 2 shows that with this transformation system we could generate kanamycin-resistant *uvh1* and C10 *A. thaliana* plants with approximately equal frequency (0.187% of the C10 seeds transformed versus 0.171% of the *uvh1* seeds transformed). Jiang et al. (1997) arrived at similar conclusions. These results further indicate that *uvh1* is not compromised in its ability to be transformed by *A. tumefaciens*.

Quantitative GUS assays with *A. tumefaciens* GV3101(pBISN1) (pBISN1 contains, as well as a *nos-nptII* gene, a *gusA*-intron gene under the control of a strong “super-promoter” [Ni et al. 1995] so that we could detect GUS activity only 2 days after infection; Nam et al. 1997) also indicated that the *uvh1* mutant was as transiently transformation-proficient as was the C10 progenitor line (Fig. 3A).

To determine directly the relative efficiencies of T-DNA integration into the genomes of *uvh1* and C10, we infected sterile root segments of these plants with the nontumorigenic strain *A. tumefaciens* GV3101(pBISN1) and grew calli derived from these roots on CIM containing phytohormones without selection for either tumors or kanamycin-resistance. These calli were used to initiate cell suspensions, composed of a mixture of transformed and nontransformed cells, grown in liquid CIM. We isolated DNA from these cells, separated high molecular weight plant DNA from any possible contaminating pBISN1 binary vector DNA by electrophoresis through a 0.6% agarose gel, and blotted the DNA onto a nylon membrane. Figure 4 shows that, when hybridized with a *gusA* gene probe, high molecular weight DNA from both *uvh1* and C10 showed similar strong signals. Control experiments indicated that this hybridization was not a result of contaminating *A. tumefaciens* DNA because hybridization to pBISN1 results in a different pattern of hybridization (Nam et al. 1997; Mysore et al. 1998; and data not shown). This result demonstrates that the efficiency of T-DNA integration into the genomes of *uvh1* and its wild-type progenitor C10 was approximately the same.

In agreement with the results of Sonti et al. (1995), we found that the mutant *rad5* was deficient in its ability to be transformed to tumors and to kanamycin-resistance. Both tumorigenesis and kanamycin-resistance in the mutant were 15% that of the wild-type progenitor, Col-0 (Figs. 1A, 1B and 3B, 3C). The tumorigenesis results were confirmed with a flower bolt inoculation assay. With this procedure, *rad5* developed much smaller tumors than did the wild-type parent, Col-0, when inoculated with *A. tumefaciens* A208 (Fig. 2). *A. tumefaciens* A348 and A281 induced small tumors on Col-0

but almost no tumors on *rad5* (Table 1). We also could not transform *rad5* to kanamycin resistance with a flower vacuum infiltration assay (Table 2). In addition, the frequency of transformation of *rad5* to ppt-resistance was lower than that of Col-0. Approximately 66% of the *rad5* root segment bundles became ppt-resistant, compared with 99% of the Col-0 root segment bundles (Fig. 3D). However, ppt-resistant calli on *rad5* were small and grew very slowly, compared with the more vigorously growing calli incited on Col-0 (Fig. 1C). In accordance with these data, the efficiency of T-DNA integration per microgram of plant DNA was approximately five times less in *rad5* than in Col-0 (Fig. 4). However, with a quantitative methylumbelliferyl- β -glucuronide (MUG) fluorimetric assay the *rad5* mutant also showed low (approximately 15% of wild-type levels) transient GUS activity (Fig. 3A), indicating that the *rad5* mutant is deficient in some step prior to T-DNA integration. We do not currently know exactly which step in the *A. tumefaciens*-mediated transformation process is disrupted in the *rad5* mutant, but a normal level of bacterial attachment to roots of this mutant (data not shown) suggests that the *rad5* mutant may be deficient in a T-DNA transfer or nuclear targeting step.

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Fig. 4. Efficiency of T-DNA integration into the genomes of *Arabidopsis thaliana* mutants *uvh1* and *rad5* and their wild-type progenitors, C10 and Col-0. Root segments of *A. thaliana* mutants *uvh1* and *rad5* and their wild-type parents C10 and Col-0 were infected with *Agrobacterium tumefaciens* GV3101(pBISN1). After 2 days of cocultivation, roots were transferred to callus-inducing medium (CIM) containing timentin (100 mg/liter) and calli grown for 4 weeks. Calli were transferred to liquid CIM containing various antibiotics to kill bacteria. After 4 additional weeks, high molecular weight plant DNA was isolated and DNA blot analysis was performed with the *gusA* gene as a hybridization probe. A photograph of the ethidium bromide-stained gel indicated equal loading of DNA in each lane (data not shown).

Table 2. Vacuum infiltration of *Arabidopsis thaliana* lines

<i>A. thaliana</i> line	Amount of seeds from vacuum-infiltrated plant germinated (mg) ^a	Kanamycin-resistant plants (no.)	Transformed seeds (%)
C10	150	14	0.187
<i>uvh1</i>	70	6	0.171
Col-0	250	7	0.056
<i>rad5</i>	250	0	<0.008

^a Ten milligrams equals approximately 500 seeds. All seed lots showed greater than 95% germination.

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