식물조직배양학회지 제27권 제5호
Korean J. Plant Tissue Culture
Vol. 27, No. 5, 387~393 (2000)

Identification of Plant Factors Involving in *Agrobacterium*-mediated Plant Transformation

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ABSTRACT The process by which *Agrobacterium tumefaciens* genetically transforms plants involves a complex series of reactions communicated between the pathogen and the plants. To identify plant factors involved in *Agrobacterium*-mediated plant transformation, a large number of T-DNA inserted *Arabidopsis thaliana* mutant lines were investigated for susceptibility to *Agrobacterium* infection by using an *in vitro* root inoculation assay. Based on the phenotype of tumorigenesis, twelve T-DNA inserted *Arabidopsis* mutants(*rat*) that were resistant to *Agrobacterium* transformation were found. Three mutants, *rat1*, *rat3*, and *rat4* were characterized in detail. They showed low transient GUS activity and very low stable transformation efficiency compared to the wild-type plant. The resistance phenotype of *rat1* and *rat3* resulted from decreased attachment of *Agrobacterium tumefaciens* to inoculated root explants. They may be deficient in plant factors that are necessary for bacterial attachment to plant cells. The disrupted genes in *rat1*, *rat3*, and *rat4* mutants were coding a arabinogalactan protein, a likely cell wall protein and a cellulose synthase-like protein, respectively.

Key words: *Agrobacterium*, *Arabidopsis*, Plant transformation, T-DNA

Introduction

Agrobacterium tumefaciens-mediated transformation is the most widely used genetic transformation system in plants (Hansen and Wright 1999). Plant transformation using *Agrobacterium tumefaciens* is a complex process, consisting of the binding of bacterial cells to the plant cell wall (Broeck and Vanderleyden 1995), induction of *vir* genes by phenolic compound released from wound plant cells (Winans 1992), the processing of a region of the Ti-(tumor inducing) plasmid and transfer this T-(transfer) DNA to plant cell (Stachel et al. 1986; Ward et al. 1988), targeting of the single-stranded T-DNA/protein complex to the nucleus (Howard and Citovsky 1990), and integration of T-DNA into plant nuclear DNA and stable expression (Gheysen et al. 1991; Mayerhofer et al. 1991). Whereas we now know considerable detail regarding the contribution of the bacterium to this process (bacterial binding to plant cell, *vir* gene regulation and function, T-DNA processing and transfer, etc.), we understand little about the plant contribution to these events.

Regarding the complete process of *Agrobacterium tumefaciens*-mediated plant transformation, obviously plant genes will contribute significantly to bacterial binding to plant cell, T-DNA targeting to nucleus and nuclear entry, conversion of the T-strand to a double-stranded form, T-DNA integration into the plant genome, and T-DNA expression (Sheng and Citovsky 1996; Zupan and Zambryski 1995, 1997). Recently several plant factors that may be involved in these processes, a karyopherin-α (Ballas and Citovsky 1997), a cyclophlin (Deng et al. 1998) and a type of 2C protein phosphatase (Gelvin, personal communication), have been identified. Other evidence for the involvement of plant factors in T-DNA transfer and integration come from idetification of several ecotypes and mutants of *Arabidopsis* that are resistant to Agrobacterium transformation (Nam et al. 1998, 1999; Mysore et al. 2000).

As a first step to identify the plant genes involving in

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Agrobacterium tumefaciens-mediated plant transformation, I have identified and characterized *Arabidopsis rat* mutants that are resistant to *Agrobacterium* infection. Consequently, three *rat* genes disrupted by T-DNA insertion in the *rat* mutants were cloned and their functions in the process of *Agrobacterium tumefaciens*-mediated plant transformation were deduced with results of DNA sequence analysis and physiological tests of *rat* mutants.

Materials and Methods

Growth of Arabidopsis plants

Seeds of Feldmann's T-DNA inserted Arabidopsis mutants pool were surface-sterilized with a solution composed of 50% commercial bleach and 0.1% SDS for 10 min, then rinsed them 5 times with sterile distilled water. The seeds were germinated in petri dishes containing Gamborg's B5 medium (GIBCO) solidified with 0.75% bactoagar (Difco). After incubation of the plates at 4 C for 2 days, we incubated them for 7 days under a 16-hr light/8-hr dark photoperiod at 25°C. Seedlings were individually transferred into baby food jars containing solidified Gamborg's B5 medium and grown for 7-10 days for root culture.

Growth of Agrobacterium tumefaciens

All *Agrobacterium* strains were cultured in YEP medium (Lichtenstein and Draper 1986) supplemented with the appropriate antibiotics (rifampicin, 10 mg/l; kanamycin, 100 mg/l) at 30°C. Overnight bacterial cultures were washed with 0.9% NaCl and resuspended in 0.9% NaCl at 2×10^9 = cfu/ml for *in vitro* root inoculation.

In vitro root inoculation and transformation assays

Roots grown on the agar surface were excised and cut into small segments (approximately 0.5 cm) in a small amount of sterile water, and blotted the root segments on sterile filter paper to remove excess water. Dried bundles of root segments were transferred to MS basal medium and 2-3 drops of the bacterial suspension 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, root bundles were infected with *A. tumefaciens* GV3101 (Koncz and Schell 1986) containing the binary vector pBISN1 (Narasimhulu et al. 1996). After various periods of time, the root segments were rinsed with water, blotted on filter paper, and stained with X-gluc staining solution (50 mM Na2HPO4, 10 mM Na2.EDTA, 300 mM mannitol, and 2 mM X-gluc, pH 7.0) for 1 day at 37 C. For quantitative measurements of GUS activity, the root segments were ground in a microcentrifuge tube containing GUS extraction buffer (50 mM Na2HPO4, 5 mM dithiothreitol, 1 mM Na2.EDTA, 0.1% sarcosyl, 0.1% Triton X-100, pH 7.0) and GUS specific activity was measured according to Jefferson et al. (1987).

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

For transformation of root segments to ppt-resistance, root bundles were inoculated with *A. tumefaciens* GV3101 containing pBISN1. After 2 days, small root bundles were transferred onto CIM containing timentin (100 mg/l) and kanamycin (50 mg/l). ppt-resistant calli were scored after 4 weeks incubation.

To determine stable GUS expression, we inoculated roots as above and transferred the root segments after 2 days to CIM containing timentin (100 mg/l) without any selection. After 4 weeks we assayed GUS activity either by staining with X-gluc or by measuring GUS specific activity using a MUG fluorimetric assay, as described above.

Bacterial adhesion to Arabidopsis roots

The roots grown as described above were chopped with a scalpel in 2 ml MS medium in a sterile 45 mm petri dish. 0.05 ml of the bacterial culture was added and incubated with the chopped roots at room temperature for 24 to 48 hours. In order to examine the roots for bacterial attachment, root pieces was removed from the

medium by draping them over a dissecting needle. The segments were suspended in a drop of water, and examined and photographed using a Zeiss photoscope 2 with Nomarski optics.

Plasmid rescue

Genomic DNA (5 mg) of *rat* mutants were isolated according to Dellaporta et al (1988) and digested to completion with *Eco*R1. Digested DNA was extracted with phenol/chloroform and precipitated with ethanol. The DNA was ligated in a final volume of 500 ml in 1x ligation buffer (Promega), 0.1 mg/ml gelatin, and 3 units of T4 DNA ligase at 16°C for 16 hr. The ligation mixture was precipitated with ethanol, transformed into *E. coli* DH5a *mcr-* by electrophoration (25 mF, 200 W, and 2.5 kV) and plated on LB medium containing ampicillin (100 mg/l). Ampicillin-resistant colonies were replica plated onto LB medium containing kanamycin (50 mg/l). Plasmids were isolated from kanamycin-sensitive colonies, digested with *Eco*RI, and the size of the digested plasmid was compared to the size of the corresponding hybridizing fragment from total genomic DNA of *rat* mutants digested with *Eco*RI and hybridized with pBR322. Recovered plant junction DNA was confirmed by detecting polymorphism between wild type and mutant in Southern hybridization probing with a putative plant junction sequence.

Screening of genomic and cDNA libraries

An *Arabidopsis* (ecotype Ws) genomic library (a gift from Richard M. Amasino, University of Wisconsin) and a cDNA library (a gift from Linda A. Castle, Oklahoma State University) were screened for the *Rat* genes using plant junction DNAs rescued from the *rat* mutants. All other nucleic acid manipulation was followed as described in molecular cloning mannual.

DNA sequencing and analysis

Genomic and cDNA clones were subcloned into pBluescript KS (-) (Stratagene), and deletions were generated using exonuclease III and S1 nuclease as described by the manufacturer (Promega). DNA sequencing was performed using an Applied Biosystems automated DNA sequencer (ALF express) using dye terminators as recommended by the manufacturer (Pharmacia). All regions were sequenced on both strands at least one time. The predicted amino acid sequence was used to search the DNA and protein sequence databases using BLAST program.

Results

Isolation of Arabidopsis mutants resistant to Agrobacterium infection

To screen for *Arabidopsis* mutant plants that showed an alteration in tumor formation after *Agrobacterium* infection, I used an *in vitro* root bundle inoculation assay. Surface-grown roots of individual three week-old T-DNA insertion-mutagenized T4 plants (Feldmann and Marks 1987; Feldmann 1991) were inoculated with *A. tumefaciens* A208. The remaining part of each plant was placed into solidified culture medium to allow root regeneration. After observing the result of the root inoculation, re-rooted plants showing a resistance response were transferred to soil and allowed to set seeds for recovery of progeny. The wild-type parent (ecotype Ws), one of the hyper-susceptible *Arabidopsis* ecotypes identified previously (Nam et al. 1997), consistently showed large green tumors with teratomas in response to *Agrobacterium* infection (Figure 1). Of approximately 3000 T4 kanamycin-resistant plants, twelve plants were found to be resistant to *Agrobacterium* infection. Three mutants, *rat1*, *rat3* and *rat4*, developed a few tumors that were significantly smaller in size compared to those incited on wild-type plants (Figure 1). These mutants were chosen for more extensive characterization.

Characterization of the rat mutants resistant to Agrobacterium tumefaciens infection

The phenotype of these *rat* mutants, *Agrobacterium*-mediated transformation resistance, was further manifested by an inability to develop phosphinothricin-resistant calli on callus inducing medium containing phosphinothricin (10 mg/l) when root segments were inoculated with the disarmed strain *A. tumefaciens* GV3101 (pCAS1). The binary vector pCAS1 contains a bialaphos resistance (*bar*) gene under the control of the *nos* promoter. Phosphinothricin (ppt) was used as a stable

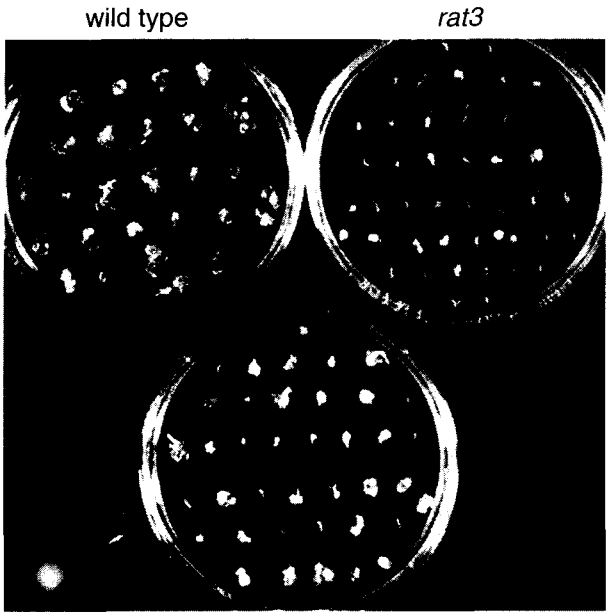

Figure 1. Stable transformation of the *Arabidopsis rat3*, the wild type progenitor Ws, and their F_1 progeny. Sterile root segments were infected with *A. tumefaciens* A208. After 2 days of cocultivation, the roots were moved to MS medium lacking phytohormones and containing timentin(100 mg/l). Crown gall tumors on root segments were induced for 4 weeks and photographed.

transformation selection marker because these *rat* mutants were already transformed with a T-DNA containing a kanamycin-resistance (*nptII*) gene.

To determine which steps in the *Agrobacterium*-mediated transformation process were disrupted in the *rat1*, *rat3* and *rat4* mutants, I compared the efficiency of T-DNA transfer to these mutant plants with that of wild-type plants by quantitatively measuring both transient GUS (ß-glucuronidase) activity and the efficiency of stable transformation (tumorigenesis and ppt-resistance). I determined the relative transient transformation efficiency by inoculating sterile root segments with *A. tumefaciens* GV3101 harboring the T-DNA binary vector pBISN1 and measuring GUS activity. pBISN1 contains a *gusA*-intron gene under the control of a super-promoter (Ni et al. 1995; Narasimhulu et al. 1996). Using this vector, I could detect GUS activity after cocultivation for only 2 days. This early expression of GUS activity most likely represents transient expression of genes harbored by T-DNA that is not yet integrated into the plant genome. The *rat1*, *rat3* and *rat4* mutants showed approximately 25% transient GUS activity compared to the wild-type plant (Table 1). In the stable transformation analyses, they showed approximately 10% tumor formation and 10% induction of ppt-resistant calli compared to the wild-type plants (Table 1). Thus, *rat1*, *rat3* and *rat4* mutants most likely have mutations that block tumorigenesis at an early stage of the transformation process.

rat1 and rat3 are deficient in Agrobacterium attachment

To examine the attachment of *Agrobacterium* to the *rat1* and *rat3* mutants, sterile root segments of these mutant plants were incubated with *A. tumefaciens* C58. Attachment assays were conducted either in water or in 0.4% sucrose. The results shows that the mutant *rat1* was highly deficient in its ability to bind *Agrobacterium*, both in water and in sucrose. The mutant *rat3* was also unable to bind *Agrobacterium* in water, however, attachment occurred in sucrose. *Agrobacterium* cells were able to attach to root segments of the wild-type *Arabidopsis* progenitor, Ws both in water and in sucrose. Thus, *atr1* and *atr3* are defective in their ability to bind *A. tumefaciens*. Somewhat surprisingly, Agrobacterium cells bind

Table 1. Stable transformation and transient GUS expression in Ws and *rat* mutants.

Mutant[a]	% of root bundles with tumors	Tumor morthology	% of root bundles with ppt-resistant calli[b]	% of root bundles stained blue with X-gluc[c]
Ws	86±15	Large, green teratomas	87 ±10	92 ±6
rat1	7± 1	Very small, yellow	5 ± 2	22 ±4
rat3	10± 4	Very small, yellow	9 ± 2	31 ±2
rat4	19± 8	Very small, yellow	14 ± 4	10 ±4

[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.
[c]At least three different plants were tested for each mutant and at least 100 root segments were observed for each plant.

to the cut surfaces of *rat4* Arabidopsis roots as well as they do to wild type roots (preliminary data).

Genetic analysis of rat mutants

To determine the genetic characteristics of *rat* mutants, each homozygous mutant plant was back-crossed to a wild-type plant. F_1 hybrid plants were selected by germinating F_1 seeds of each cross on Gamborgs B5 medium containing kanamycin (50 mg/ml). All F_1 hybrid plants displayed an intermediate response between the mutant and wild-type in the tumorigenesis and ppt-resistance assays (Figure 1) In the F_2 population, the kanamycin-resistance phenotype segregated as a single dominant characteristic (kan^r : kan^s = 3 : 1) indicating that a single linkage group was disrupted by T-DNA insertion in each mutant, although the number of T-DNAs integrated in each mutant could be different. To examine the co-segregation of the T-DNA insertion with the *Agrobacterium* resistance phenotype, individual F_2 plants were grown on solidified Gamborgs B5 medium without kanamycin. Root bundles were infected with *A. tumefaciens* A208 and transferred onto MS basal medium to induce tumors. The remaining part of the plant was transferred onto Gamborgs B5 medium containing kanamycin (50 mg/ml) to screen for kanamycin-sensitive (k/k) plants. Kanamycin-resistant plants were transferred into soil to obtain F_3 seed. Seeds from each F_2 plant were germinated on Gamborgs B5 medium containing kanamycin (50 mg/ml) to determine the genotype of kanamycin-resistant plants among the F_2 progeny. In these F_2 populations, the genotype of kanamycin-resistance and tumorigenesis segregated as 1:2:1 (k/k:K/k:K/K) and 1:2:1 (susceptible : intermediate : resistant), respectively. If the *rat1*, *rat3* and *rat4* mutants were semidominant, all F_2 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. It was found that F_2 plants sensitive to kanamycin (k/k) were uniformly as susceptible as were the wild-type parent. However, F_2 plants (K/k and K/K) resistant to kanamycin did not segregate with the expected phenotype. Some heterozygous plants (K/k) showed a resistance phenotype similar to that of homozygous plants (K/K). In contrast, some homozygous plants (K/K) showed an intermediate phenotype similar to that of heterozygous plants (K/k). This confusing results may derive from difficulty in distinguishing between the intermediate and resistant tumorigenesis phenotype. However, the clear co-segregation of kanamycin-sensitivity (k/k) with the susceptible phenotype, and the lack of susceptible plants homozygous for kanamycin-resistance, indicated that the phenotypes of *rat1*, *rat3* and *rat4* mutants are linked to the locus into which the T-DNAs integrated in each *rat* mutants. These data indicate that *rat1*, *rat3* and *rat4* are semidominant mutants resulting from integration of T-DNA into a single genetic locus, and that these genes are linked by the T-DNA.

Cloning of genes disrupted in rat *mutants*

Plasmid rescue experiments isolated T-DNA/plant DNA junction regions from *rat1*, *rat3* and *rat4* mutants, and subsequently wild type genes disrupted in the *rat* mutants were identified. DNA sequence analysis of these genes indicated that *rat1* encodes an arabinogalactan protein(AGP) and *rat3* encodes a small protein that is likely secreted to the apoplast. AGPs are clearly often present in the extracellular space in plant, and their function, like that of the glycosaminoglycans of animal tissue, may well be in cell-cell adhesion, communication (Knox 1999). The involvement of AGPs in

Table 2. Co-segregation analysis of *rat* mutants

Mutants	Number of plant tested	Phenotypes		x^2 value[a]	Cromosome localization
		Kan^r/Tum^-	Kan^s/Tum^+		
rat1	44	30	14	1.09*	2
rat3	50	42	8	2.16*	5
rat4	98	70	28	0.67*	5

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

Agrobacterium-mediated transformation was further confirmed using ß-glucosyl Yariv reagent, which binds AGPs specifically. When Arabidopsis root segments were incubated with an active Yariv reagent prior to inoculation with Agrobacterium, transformation was blocked. An inactive ß-mannosyl Yariv reagent, however, did not block transformation. Control experiment indicated that ß-glucosyl Yariv reagent did not affect the viability of Arabidopsis root segment or Agrobacterium cells. Another *rat* gene, *Rat4*, encodes a cellulose synthase (*CelA*)-like protein. Analyses of expression sequence tags(ESTs) of *celA* and *celA*-like genes in Arabidopsis indicated that there might be as many as 40 members of these gene family in Arabidopsis. (T Richmond, P Villand, S, Cutler, and C Somerville, poster presented at the 9th International Conference on *Arabidopsis* Research, Madison, WI 1998).

Discussion

Using *Arabidopsis thaliana* as a model plant in the research of interaction between *Agrobacterium* and plant, it was possible for the first time to isolate *Arabidopsis* mutants showing a polymorphism in susceptibility to *Agrobacterium* infection. Most of the *rat* mutants identified to date show the same phenotype. In particular, *rat1*, *rat3*, and *rat4* are highly recalcitrant to both stable and transient transformation, suggesting that they are blocked at an early step in the *Agrobacterium*-mediated transformation. This hypothesis is in accord with the results that the *rat1* and *rat3* mutants are deficient in binding *Agrobacerium* to their roots. This lack of attachment in water suggests that there is a surface alteration in these plants. The finding that the *Rat1* and *Rat3* genes encode an arabinogalactan protein and cell wall protein, respectively is consistent with this hypothesis. Interestingly, *rat4* mutant disrupted *CelA*-like gene shows similar phenotypes of *rat1* and *rat3*, but is normal in binding *Agrobacterium* to its root segments. This results suggest that *rat4* mutant may be deficient in other early steps in the *Agrobacterium*-mediated transformation, such as T-DNA transfer from *Agrobacterium* to plant cell and T-DNA translocation to nucleus in plant cell. More sophisticated analytical methods need to be developed first to distinguish these steps *in vivo*. Another question remaining to be addressed is how mutation of just this one member of such a large multigene family can abolish Agrobacterium-mediated transformation.

Isolation and genetic analysis of mutants, and subsequent cloning of affected genes will lead to significant advances in our understanding of how Agrobacterium transform plant efficiently. A detailed functional analysis of these genes will help us not only determine why plants differ in susceptibility to *Agrobacterium*-mediated transformation but also understand how plant carry out basic cell biology processes such as cell wall biosynthesis, protein and nucleic acid targeting to the nucleus, and repair and recombination of nuclear DNA. This is true because it is likely that *Agrobacterium* has borrowed the plant molecular machinery to carry out each of these processes necessary for transformation. Practically, the isolation and analysis of plant genes necessary for *Agrobacterium*-mediated transformation may allow us to construct new bacterial strains and agronomically important host plants that are more susceptible to T-DNA mediated genetic transformation.

Acknowledgements **:** This work was supported by the Dong-A University Research Fund, in 2000 and grant No 2000-2-21000001-3 from the Basic Research Program of the Korea Science & Engineering Foundation.

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