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# Molecular cloning of the *Arabidopsis* gene *rat3* that is involving in the *Agobacterium*-mediated planttransformation

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## Abstract

Genomic and cDNA clones containing the *RAT3* gene involving in *Agrobacterium*-mediated plant transformation were identified using plant DNA flanking the right border of a T-DNA rescued from the *rat3* mutant as a hybridization probe. Two highly homologous cDNA clones were identified; one (*RAT3-1*) weakly hybridized with the probe, whereas another (*RAT3-2*) strongly hybridized with the probe. Both Rat3-1 and Rat3-2 proteins contain a putative signal peptide for secretion. The deduced molecular weights of encoded proteins are 15 kDa. The results of genomic DNA blot analysis and DNA sequencing indicated that *RAT3-1* and *RAT3-2* exist as single copy genes and they were arranged side by side with just 600 bp distance between them. *RAT3-1* was disrupted by the integration of T-DNA into the 3′ untranslated region in *rat3* mutant. A BLAST search showed that both RAT3-1 and RAT3-2 proteins have homology with only the C-terminal region of $\beta$-1,3-glucanase homologues from *Triticum aestivum* and *Arabidopsis thaliana*. These $\beta$-1,3-glucanase homologues contain an unusually long C-terminal region with no significant homology to other $\beta$-1,3-glucanases.

**Key words** – Agrobacterium tumefaciens, crown gall tumors, plant transformation

## Introduction

Despite the relevance of *Agrobacterium tumefaciens* as a plant pathogen and as an important genetic engineering tool, little is known about the molecular events that take place in the host cell during genetic transformation by *Agrobacterium*. Specifically, there is a lack of knowledge of the plant factors and their genes involved in bacterial attachment, T-DNA transfer, nuclear import of the putative T-complex, and T-DNA integration [1-3]. As a first step to identify such plant genes, we have identified and characterized *Arabidopsis rat* mutants resistant to *Agrobacterium*-mediated transformation [4,5]. The mutant line *rat3* was chosen as a starting point to clone the corresponding *RAT3* gene from the wild-type plant because this mutant showed the simplest T-DNA integration pattern. In addition, we showed genetically that the mutant phenotype in *rat3* mutants was linked to the T-DNA insertion [5]. The goal of this work was to clone the *RAT3* gene disrupted by T-DNA insertion into the *rat3* mutant and to attempt to deduce its function in the *Agrobacterium* infection process through DNA sequence analysis.

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## Material and Methods

### Nucleic acid manipulation

Total plant genomic DNA was isolated according to the method of Dellaporta [6]. Restriction endonuclease digestions, agarose gel electrophoresis, plasmid isolation

were conducted as described [7].

### Plasmid Rescue

Genomic DNA (5 $\mu g$) of *rat3* mutant was 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 $\mu\ell$ 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). Kan$^r$ colonies are likely to contain the 7.2 kbp self-ligated plasmid consisting of the left portion of pBR322, Tn*903*, and Tn5 sequences (Fig. 1. A). Tn*903* confers Kan$^r$ upon *E. coli*. 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 *rat3* digested with *Eco*RI and hybridized with pBR322.

### 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 RAT3 gene using plant junction DNA rescued from the *rat3* mutant. *E. coli* LE392 (for the genomic library) or C600 (for the cDNA library) were grown to log phase in Luria Broth (LB) supplemented with 0.2% maltose and 10 mM $MgSO_4$. For each plating, 0.5 ml of host *E. coli* cells were mixed with an amount of phage from each library that would

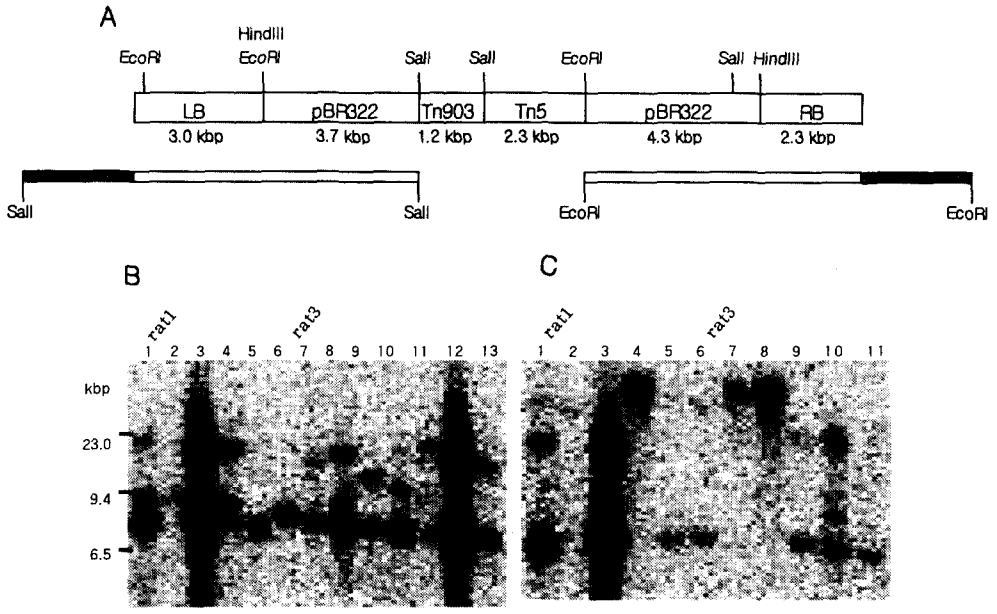


Fig. 1. T-DNA integration patterns and analyses of right and left border regions in the *rat* mutants.
The 3850::1003 T-DNA (A) Feldmann used to generate *Arabidopsis* T-DNA insertion mutant lines contains two copies pBR322 near the right and left borders. When genomic DNA of each mutant plant was digested with *Eco*RI for right border analysis (B) or *Sal*I for left border (C) and hybridized with pBR322 sequence, at least two hybridizing bands could be detected. One is the internal pBR322 sequence, 7.2 kbp and 6.7 kbp for *Eco*RI and *Sal*I digestions, respectively. The others are right (or left) pBR322 sequence+right (or left) border sequeces+plant DNA whose size is dependent on the location of *Eco*RI or *Sal*I sites in the flanking plant DNA or internal T-DNA sequences in the case of multiple T-DNA integration into same site. Each number on the top of the gel indicates a putative mutant lines initially identified.

generate approximately $2\times10^4$ plaque forming units (pfu) per 150 mm plate. The mixture of *E. coli* and phage was incubated at 37°C for 15 minutes with gentle shaking to allow the phage to absorb onto the cells. After incubation, the infected *E. coli* were added to 8 ml of 0.7% top agarose, mixed gently, and poured onto prewarmed 150 mm agar plates. These plates were incubated at 37°C for 12 hours to allow for the phage to lyse the *E. coli*, leaving visible plaques. To these plates, 132 mm nylon membrane filters were overlaid on top of the plaques. The orientation of the filters was determined by ink marks. After one minute, the filters were removed from the plates and placed in a tray containing denaturing solution (1.5 M NaCl, 0.5 M NaOH) for one minute. They were transferred to neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl [pH 8.0]) for five minutes, then rinsed in 2×SSPE (18 mM NaCl, 10 mM $NaH_2PO_4$, 1 mM $Na_2EDTA$). After drying, the filters were exposed to UV light to link the DNA to the filter, and the filter was hybridized with the labeled probe and processed as described [8].

After autoradiography, we aligned the film with the plates at the ink marks and isolated a small piece of agar containing potential positive plaques. The phage were isolated from the agar by soaking in SM buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgSO_4$, and 0.2% gelatin) and plated to obtain single positive plaques.

### DNA sequencing and analysis

Genomic and cDNA clones were subcloned into p Bluescript 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). The predicted amino acid sequence of RAT3 was used to search the DNA and protein sequence databases using BLAST. The alignment of $\beta$-1,3-glucanase sequences was generated using the program Pileup.

## Results

### T-DNA integration patterns in *rat* mutants

To determine the number of T-DNAs integrated in each of the *rat* mutants, DNA gel blot analysis of the *rat* mutant plants was performed using as a hybridization probe the large fragment of pBR322 digested with *Eco*RI and *Sal*I. Three mg DNA from each mutant plant was digested with *Eco*RI for left border or *Sal*I for right border analysis. Because the T-DNA in the Ti-plasmid used by Feldmann [8,9] contains two copies of pBR322 flanking the *nptII* gene, at least two hybridizing bands should be detected using either restriction endonuclease (Fig. 1. A). One band contains internal pBR322 sequences, 7.2 kbp or 6.7 kbp for *Eco*RI digestion or *Sal*I digestion, respectively. The other fragment contains the right (or left) pBR322 sequence plus the right (or left) border sequence plus plant DNA. The size of this fragment depends on the location of the *Eco*RI or *Sal*I site in the flanking plant DNA. By comparing the number, size, and intensity of hybridizing bands, the number of T-DNAs integrated in the mutants can be determined. For example, only one T-DNA was integrated in the *rat3* mutant because two hybridizing bands were detected and each band had approximately the same intensity (Fig. 1. B). However, multiple T-DNAs were integrated into the genome of the *rat1* mutant (Fig. 1. B).

### Recovery of plant DNA flanking the T-DNA insertion in the *rat3* mutant by plasmid rescue

DNA blot analysis indicated that there was a single T-DNA insertion in *rat3*. The recovery of the right border had some advantages over recovery of the left border. These included the smaller size of the self-ligated plasmid, easy distinction from internal pBR322 sequences using antibiotic selection (the border fragment-containing plasmid would be ampicillin-resistant and kanamycin-sensitive,

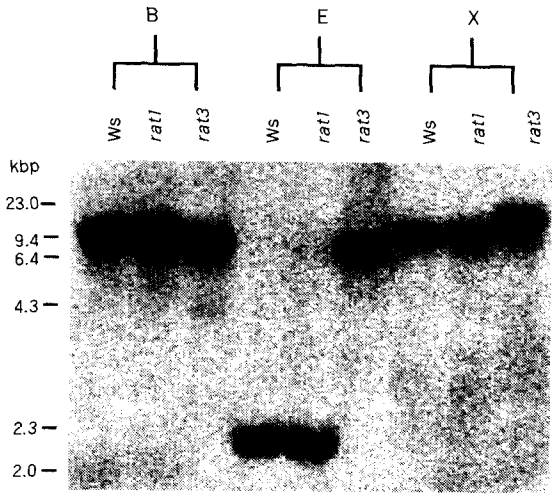


Fig. 2. DNA gel blot analysis of genomic DNA from the *rat1*, *rat3*, and wild type plants.
genomic DNA of wild type, *rat1*, and *rat3* plants were digested with several restriction endonucleases [*Bam*HI (B), *Eco*RI (E), and *Xba*I (X)], and hybridized with the RAT3-1 cDNA. Restriction fragment length polymorphism (RFLP) between the wild-type and the *rat3* mutant resulted from a disruption of *rat3* locus by the T-DNA insertion in the *rat3* mutant.

whereas the internal T-DNA fragment would be both ampicillin- and kanamycin-resistant), and the relatively more precise integration usually associated with the right border repeat sequence relative to the left border repeat sequence [10,11].

Two identical plasmids showing the same size *Eco*RI fragment as that revealed by genomic DNA gel blot hybridization (7.8 kbp) were obtained by plasmid rescue. This plasmid was used to probe DNA gel blots of wild-type *Arabidopsis* DNA to confirm that they contained plant DNA. The plasmid consists of a 4.3 kbp pBR322 sequence, 2.3 kbp right border sequence, and 1.2 kbp of plant DNA. The *Sac*II-*Eco*RI fragment containing 150 bp of right border sequence and the joining 1.2 kbp of plant DNA was sequenced to identify the T-DNA integration site. The T-DNA was integrated from 8 bases upstream of the VirD2 nicking site of the right border repeat sequence and 11 nucleotides of unidentified sequence (filler DNA) was inserted between the T-DNA and the plant DNA (data not shown).

### Isolation of *RAT3* genes

Plant junction DNA isolated from the rescued plasmid was used to probe an *Arabidopsis* (ecotype Ws) genomic library (gift of Richard M. Amasino, University of Wisconsin) and a cDNA library (gift of Linda A. Castle, Oklahoma State University) constructed in λGEM11 and λgt10, respectively. Two homologous but non-identical cDNAs were identified. One (*RAT3-1*) showed a weak hybridization signal when hybridized with the junction DNA. Another one (*RAT3-2*) showed a strong hybridization signal. Both *RAT3-1* and *RAT3-2* cDNAs were subcloned into pBluescript KS (-) and sequenced. They showed approximately 70% amino acid sequence homology (Fig. 3. C). The 1.2 kbp right border junction DNA contained exactly the same sequence as did the *RAT3-2* cDNA, indicating both RAT3-1 and RAT3-2 are located at very close in the Arabidopsis genome (Fig. 3. A). This result was confirmed by sequencing the genomic clone.

In addition, to confirm that these cDNAs derive from plant DNA flanking the T-DNA insertion site in the *rat3* mutant, genomic DNAs from wild-type, *rat1*, and *rat3* plants were digested with several restriction endonucleases and hybridized with the *RAT3-1* cDNA. Restriction fragment length polymorphism (RFLP) was found between the wild-type and the *rat3* mutant but not between wild-type and the *rat1* mutant, indicating that this locus was not disrupted by the T-DNA insertion in the *rat1* mutant (Fig. 2). This result also suggests that *rat1* and *rat3* are not alleles because their disrupted genes resulting in mutant phenotype in these mutant are different.

### Structure of the *RAT3-1* and *RAT3-2* genes

The genomic DNA (4.7 kbp) including the *RAT3-1* and *RAT3-2* cDNAs was sequenced, and the exact locations of *RAT3-1* and *RAT3-2* were identified (Fig. 3,

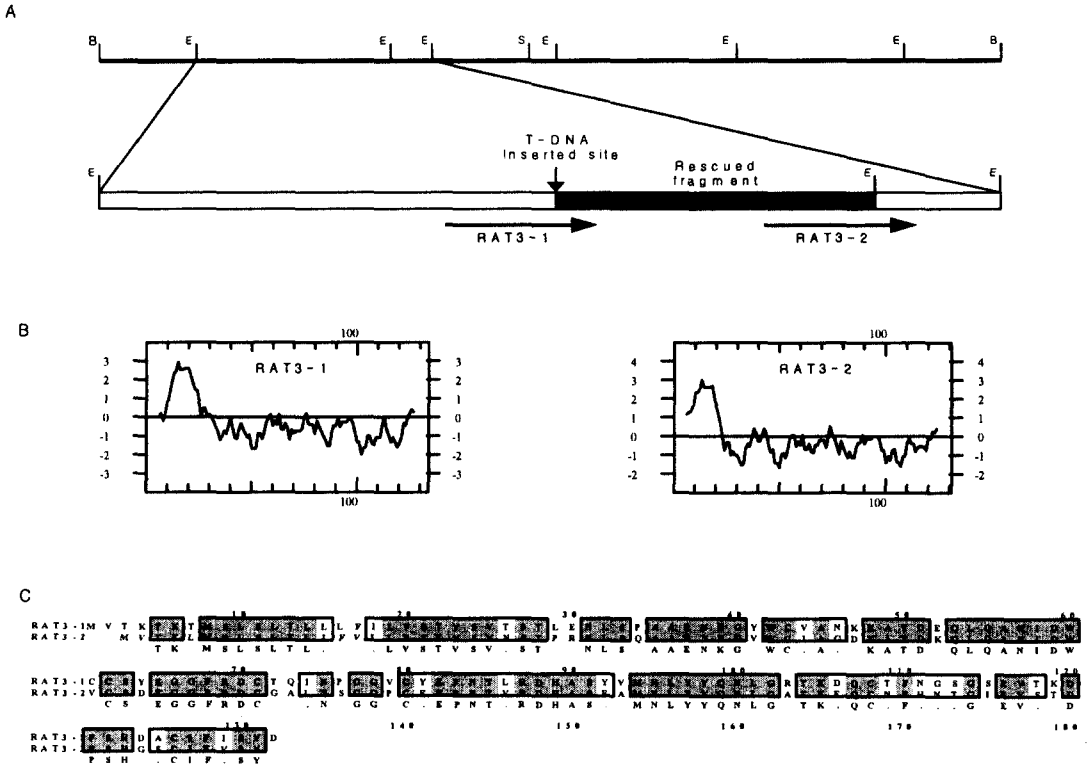


Fig. 3. Structure of the RAT3 genomic and cDNA clones.

(A) RAT3 genomic clone showing *Bam*HI (B), *Eco*RI (E), and *Sal*I (S) restriction sites. The magnified region displayed below the genomic clone (*Eco*RI fragments) specifies the location of the T-DNA insertion and the plant genomic DNA fragment isolated by right border rescue. The arrows below the magnified genomic region indicate the exact location of two homologous genes, RAT3-1 and RAT3-2 and their orientations of transcription. No intron was found in either of the genes.

(B) Profile of hydrophobicity of the RAT3-1 and RAT3-2 proteins. Both proteins have predicted signal peptides at the N-terminal region. The proteins are very hydrophilic (weak acid)

(C) Alignment of the deduced amino acid sequences of RAT3-1 and RAT3-2. Identical residues or functionally similar residues are boxed in black and gray, respectively.

A). The two genes are arranged tandomly with 600 bp distance between them. No intron was found in either gene. The deduced molecular weight is 15 kDa for each protein. Both *RAT3-1* and *RAT3-2* gene products contain a putative N-terminal hydrophobic signal peptide for secretion (Fig. 3, B). The *RAT3-1* gene was disrupted by the T-DNA insertion in the 3′ untranslated region in the *rat3* mutant (Fig. 3, A). Although *RAT3-1* and *RAT3-2* cDNAs do not contain the consensus polyadenylation signal sequence (AATAAA/T) at the 3′ region, they have the putative polyadenylation signal, AATAAG located 36 and 51 nucleotides downstream from their respective termination codons. The genomic sequence revealed that both *RAT3-1* and *RAT3-2*genes have the putative TATA box at 36 and 32 nucleotides upstream region from their respective 5′ ends of the largest cDNAs, even though we do not know the transcription initiation sites in these genes (data not shown).

A BLAST search showed that the RAT3-1 and RAT3-2 protein sequences have homology to $\beta$-1,3-glucanase homologues from *Triticum aestivum* and *Arabidopsis thaliana*. Figure 4. shows an alignment of the RAT3-1 and RAT3-2 protein sequences with $\beta$-1,3-glucanase homologues from *Triticum aestivum* (Ta BGH) and *Arabidopsis* (At A6) and

a typical pathogen inducible $\beta$-1,3-glucanase from *Arabidopsis* (At BG2). RAT3-1 and RAT3-2 protein sequences have homology with only the C-terminal region of the $\beta$-1,3-glucanase homologues from *Triticum* and *Arabidopsis*. These particular $\beta$-1,3-glucanase homologues have unusually long C-terminal regions.The RAT3-1 and RAT3-2 proteins do not have homology with At BG2 and putative catalytic domains of $\beta$-1,3-glucanase (Fig. 4).

## Discussion

To isolate the disrupted gene in *rat3* mutant that is deficient in bacterial attachment, We used a plasmid rescue technique to isolate plant DNA flanking the T-DNA right border insertion from the *rat3* mutantBy using this flanking DNA, genomic and cDNA clones of the *RAT3* gene from wild-type *Arabidopsis* (ecotype Ws) were identified.

Sequencing of two similar cDNA clones (*RAT3-1* and *RAT3-2*) and a genomic DNA clone revealed that there is approximately 70% identity between amino acid sequences but no significant homology between the 3′ untranslated regions of *RAT3-1* and *RAT3-2* genes (Fig. 3. C and data not shown). These two genes are located very close to each other in the genome, with only 600 bp distance between them. Such a clustering of the functionally related genes are commonly found in the plant genome. Specially, many disease resistance (*R*) genes, such as *Mla* locus of barley and the *Rp1* locus of maize and *Pto* gene family, have been shown to be clustered [12,13].

The *RAT3-1* gene was disrupted by the T-DNA insertion in the *rat3* mutant. It may be difficult to generate a mutant by T-DNA tagging of a gene that has multiple copies because of the need to inactivate all copies of the gene to bring about loss of gene function. If the

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                                                    a
At BG2   225   TVDN-DPGLYSQNLFDANLDSVYAALEKSGGGSLEIVVS ETGWPTEGAVG-TSVE--NAK  280
At A6    242   TYTDPQTGLVYRNLLDQMLDSVLFAMTKLGYPHMRLAIS ETGWPNFGDIDETGANILNAA  301
Ta BGH   226   GIYDPATKLNYTSMLDAQMDAIYTAMKKLGYGDVDIAVG EAGWPTQAEPGQIGVGVQEAR  285
RAT3-1
RAT3-2

                                                    b
At BG2   281   TYVNNLIQHVKNG----SPRRPGKAIETYIFAMFD ENKKE-PTYERFWGLFHPDRQSKYE  335
At A6    302   TYNRNLIKKMSASPPIGTPSRPGLPIPTFVFSLFN ENQKSGSGTQRHWGIFDPDGSPIYD  361
Ta BGH   286   DFNEGMIR-VCSSGKG-TPLMPNRTFETYLFSLFD ENQKPGPIAERHFGLFNPDFTPVYD  243
RAT3-1
RAT3-2

At BG2   336   VNFN                                                          339
At A6    362   VDFTGQTPLTGFNPLPKP TNNVPYKGQVWCVPVEGANETELEETLRMACAQSNTTCAALA  421
Ta BGH   244   LGLASVAPTPSPNPSPNP SPPAPSGGGKWCVAKDGANGTDLQNNINYACGF-V-DCKPIQ  301
RAT3-1   18    -ILVSTVSVTST--LENLSPAAENKGY-WCVANKKATDEQLQANIDWCCSYGGFDCTQIN  73
RAT3-2   15    VLLVSTVSVNST-PR-NLSQAAENKGV-WCIAGDKATDKQLQANIDWVCSDEGGFCGALN  71

AtBG2
At A6    422   PGRECYEPVSIYWHASYALNSYWAQFRNQSIQCFFNGLAHETTTN-PGNDRCKFPSVTL   479
Ta BGH   302   SGGACFSPNSLQAHASYVMNAYY-QANGHTGLACGFKGTGIVTSSDPSYGGCKV-S      355
RAT3-1   74    PGGVCTEPNTLRDHASYVMNLYY-QNLGRTKDQCTFNGSGSEVTKDPSHDACIFISYD   132
RAT3-2   72    SGGPCFEPNTVRDHASFAMNLYY-QNLGATKEQCNFHNTGIEVSTDPSHGSCIFVS     129
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Fig. 4. Sequence alignment of the deduced RAT3-1 and RAT3-2 proteins with several $\beta$-1,3-glucanases.
The sequences compared are At BG2, a pathogen inducible $\beta$-1,3-glucanase from *Arabidopsis*; At A6, an anther-specific $\beta$-1,3-glucanase homologue from *Arabidopsis*; Ta BGH, a $\beta$-1,3-glucanase homologue from *Triticum aestivum*. Regions a and b are putative catalytic domains.

*rat3-2* gene is also a functional gene, a possible explanation for the phenotype of the *rat3* mutant is the inactivation of both *RAT3-1* and *RAT3-2* genes by a single T-DNA integration event. T-DNA is inserted into the 3′ untranslated region of the *RAT3-1* gene. This insertion site is very close to the putative promoter region of the *RAT3-2* gene. Thus, *RAT3-1* mRNA may be unstable or untranslatable due to disruption of the 3′ untranslated region, and the transcription of the *rat3-2* gene may be inhibited by the insertion of T-DNA so close to the putative promoter region of *RAT3-2* gene. To test this hypothesis we need to perform RNA gel blot analyses using *RAT3-1* and *RAT3-2* gene-specific probes.The 3′ untranslated regions of these genes do not have significant homology with each other, and should be useful in this analysis. In addition, complementation of the *rat3* mutant with each of these genes will be necessary.

When compared with sequences in the GenBank database, RAT3-1 and RAT3-2 show significant homology with only the C-terminal region of $\beta$-1,3-glucanase homologues from *Triticum* and *Arabidopsis* (BLASTX scores 180 and 130, respectively). The $\beta$-1,3-glucanase homologue from *Arabidopsis*, the *At A6*, gene, is tapetum-specific and temporally expressed with a peak in activity when the plant normally expresses callase (a complex of endo- and exo-$\beta$-1,3-glucanase activities) [14]. These activities are developmentally regulated and not inducible as a defense response. This glucanase activity is responsible for dissolution of the tetrad callose wall and the release of the young microspores into the anther locules [14]. However, another anther-specific glucanase, Tag1 from tobacco, has 42% and 37% DNA and protein sequence identity to At A6, respectively, and does not have a C-terminal extension [15]. Although the function of C-terminal regions of $\beta$-1,3-glucanase homologues from *Arabidopsis* and *Triticum* is not known, it is probably not related to anther specificity, because the $\beta$-1,3-glucanase homologue from *Triticum* was reported to be associated with aluminium toxicity in wheat root [16]. Phylogenically, the At A6 protein is significantly divergent from other members of the $\beta$-1,3-glucanase family due to the unusual long C-terminal region that is lacking in other $\beta$-1,3-glucanase proteins [14]. *RAT3* gene products might not have glucanase activity because of lack of homology with the conserved catalytic domains found among other members of the $\beta$-1,3-glucanase family [17].

The *rat3* mutant showed low transient GUS transformation activity and very low stable transformation efficiency, indicating that *rat3* mutant might be deficient in an early step of the *Agrobacterium*-mediated transformation process. Thus, the function of the *RAT3* gene products may be to promote bacterial attachment to the plant cell or T-DNA transfer from the bacterium to the plant cell. The *RAT3* gene products may play a role at the plant cell membrane or wall. In accordance with this hypothesis, the deduced sequences of the RAT3 proteins contain a putative signal peptide for secretion and a putative N-glycosylation sequence (N-X-S/T). The mature forms of the putative RAT3 proteins are very hydrophilic, and a putative transmembrane domain is not present. In addition, we have not found sequences indicative of prenylation or myristylation sites, common modifications that often determine membrane location [19,20]. Taken together, these data suggest that the RAT3 proteins may be secreted into the apoplast and may function in theplant cell wall rather than in the membrane. The exact mechanism of involvement of RAT3 in Agrobacterium attachment to plant tissue warrants further investigation.

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## 초록 : *Agrobacterium*에 의한 식물형질전환에 관여하는 *Arabidopsis* RAT3 유전자의 분리와 분석

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*Agrobacterium*에 의한 식물형질전환은 가장 일반적으로 많이 사용되는 식물형질전환 기술이나 이 과정에 관여하는 식물 유전자들에 대한 연구는 거의 전무한 상태다. 본 연구는 *Agrobacterium*의 감염에 저항성을 보이는 새로운 돌연변이들의 분리와 분석 연구에 연속하여 *Agrobacterium*에 의한 식물형질전환에 관여하는 *Arabidopsis* RAT3 유전자의 cDNA와 gemonic clone을 plasmid rescue 기술을 이용하여 분리하였다. 염기서열 분석결과, 매우 유사한 2개의 유전자가 (RAT3-1과 RAT3-2) 약 600 bp 간격을 두고 연속하여 존재함을 밝혔다. 그중 RAT3-1이 mutagen으로 사용된 T-DNA에 의해 손상을 받아 *rat3* 돌연변이 형질이 유도되었다. RAT3 유전자의 단백질의 분자량은 15 kDa 정도이며 아미노 말단에 분비를 위한 signal peptide를 가지며 단백질이 전체적인 매우 친수성인 것으로 미루어 세포막 밖으로 분비될 것으로 생각된다. 이들 유전자의 정확한 생물학적 기능에 대한 연구들이 수행 중이며, 이러한 기초연구는 식물형질전환 기술의 개발에 기여할 것이다.