Characterization and Host Range Determination of Soybean Super Virulent Agrobacterium tumefaciens KAT23

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Agrobacterium tumefaciens KAT23 isolated from peach root causes crown gall disease in a number of grain legume plants, including the common bean (Phaseolus vulgaris) and soybean (Glycine max). KAT23 caused tumor formation in each of these plants more effectively than strain C58. Biotype determination suggested that this strain is biotype II. KAT23 was able to utilize nopaline as a carbon source. Partial sequence analysis indicated that KAT23 harbors a nopaline-type Ti plasmid, designated pTiKAT23, which was highly homologous with other nopaline-type Ti plasmids (pTiC58 and pTiSAKURA).

KAT23 transferred not only the T-DNA of the Ti plasmid but also introduced T-DNA of the binary vector efficiently. The common bean inoculated with KAT23 (pIGFP121-Hm) showed crown galls, and some plants showed β-glucuronidase (GUS) and sGFP (S65T) gene expression. This virulent ability of KAT23 indicates its potential application to legumes, especially to soybean transformation.

Key words: Agrobacterium tumefaciens; soybean; transformation; Ti plasmid

Agrobacterium tumefaciens is a bacterial species causing crown gall disease in a wide host range of plant species. It infects the plants and forms enlarged tumors at the wounded site. The transferred DNA (T-DNA) that segments of Ti plasmid harbor in A. tumefaciens mediates this tumorogenesis. This segment encodes oncogenes, auxin biosynthesis genes, and the cytokinin biosynthesis gene, which induce tumor formation in the plant as a result of uncontrollable cell division. T-DNA also encodes the synthesis pathway of the unusual amino acid compounds opine, which is produced in tumors.

Since A. tumefaciens has opine catabolic genes on Ti plasmid in a position outside of T-DNA, it preferentially utilizes opine in tumor cells of the host plant. These opine synthesis or catabolism properties are referred in order to classify the A. tumefaciens into certain groups. The large numbers of A. tumefaciens strains are divided mainly in two opine type groups, octopine or nopaline, and each type of A. tumefaciens strain has a Ti plasmid which contains the synthesis and catabolism genes for the corresponding opine. Common octopine-type Ti plasmid has two separated T-DNA segments, and nopaline-type Ti plasmid has a single T-DNA segment. These T-DNA regions are delimited by 25 bp of imperfect direct repeats, called the right and left border sequences at both ends. This structural difference is important to the strategy of obtaining the disarmed strain of A. tumefaciens. In addition, A. tumefaciens strains are classified by physiological and biochemical tests, and in this way A. tumefaciens groups are divided into three biotypes.

T-DNA transfer is mediated mainly by the function of the vir genes (virulence genes), which form a gene cluster in the region (vir region) beside T-DNA on the Ti plasmid. This function involves (i) single strand T-DNA excision, (ii) prevention of DNase degradation, (iii) transport into the plant cell from the bacterial cell, and (iv) a pilot protein to the plant nucleus. The subsequent stable integration of T-DNA into the plant genome is achieved by an unknown pathway.

An A. tumefaciens-mediated plant transformation system was designed by applying this DNA delivering ability from microorganism to higher plant using a binary vector. The helper Ti plasmid lacks the abilities for oncogenesis but retains the full vir functions to transfer T-DNA by removing the entire T-DNA. Binary
vector has a wide host range vector and contains an alternative T-DNA region among the left and right border sequences, so the *vir* functions of the helper Ti plasmid act in transit, and T-DNA on the binary vector is transferred to the plant.\(^{11}\)

Since some famous disarmed *A. tumefaciens* strains (*viz.*, EHA105) are supplied now and researchers need only to heed this for construction of the binary vector, this means that improvement of *A. tumefaciens* strains has not been much researched.

Soybean is an important crop for protein and oil sources, but molecular biological analysis of soybean falls behind other crops due to the difficulty of obtaining transgenic plants. To our knowledge, this is due to the fact that soybean shows difficulty in *in vitro* tissue culture and recalcitrance for *A. tumefaciens* infection. Many researchers have devoted long experimental effort to obtain a transgenic soybean and have reported optimized co-cultivation conditions to ensure *A. tumefaciens* infection, specific cell induction from host plants, and selection and regeneration from transformed cells.\(^{12-16}\) However, these results are limited to a few soybean cultivars, and appear to be strongly affected by differences between the recalcitrance of soybean cultivars for *A. tumefaciens* infection. Hence we have developed a strategy to utilize the wide host range of soybean transformation.

This study is the first step in the development of *A. tumefaciens*-mediated soybean transformation using the novel soybean super virulent *A. tumefaciens* strain. We have performed screening of legume super virulent *A. tumefaciens*. Kaku *et al.* isolated 20 strains from peach root and confirmed their ability to infect and cause crown gall tumor formation in the common bean (*Legulus* and Minidoka), soybean (*Peking*, *Harosoy*, Enrei, and Suzuyutaka) in 1999.\(^{17}\) One of these strains, AT96-6, showed crown gall formation with all cultivars of common bean and soybean. We renamed this strain KAT23 and explored its availability as legume (especially soybean) super virulent *A. tumefaciens*. And we paid attention to this strain’s properties in exploiting the novel soybean transformation system.

**Materials and Methods**

**Plant materials and culture conditions.** All soybean seeds were propagated in a greenhouse, and common bean seeds were obtained from a local market. Plants were usually grown on 10 cm sawdust balls or autoclaved soil in 20 cm Wagner pots in a growth chamber at 28 °C under a 16-h light condition.

**Bacterial strains and culture conditions.** All *E. coli* and *A. tumefaciens* strains were kept as 30% glycerol stocks at −70°C for long-term storage. *E. coli* strains were cultured in Luria Broth containing appropriate antibiotics at 37 °C. *A. tumefaciens* strains were cultured in Luria Broth or on LB agar at 25 °C. From preexper-imental results of *A. tumefaciens* culture, the absorbance of liquid cultured medium was defined as OD\(_{600}\) = 0.3 for middle growth culture (3 × 10\(^8\) cells/ml), and as OD\(_{600}\) = 0.9 for full-growth culture (1 × 10\(^{14}\) cells/ml) (unpublished data).

**DNA isolation and bacterial transformation.** Basic DNA manipulations of *E. coli* were performed according to molecular cloning.\(^{18}\) Total DNA isolation from *A. tumefaciens* was done according to the bacterial genomic DNA isolation procedure.\(^{19}\) Introduction of the binary vector into *A. tumefaciens* was carried out by electroporation.\(^{20}\) *A. tumefaciens* was cultured in 10 ml of Luria Broth with shaking in an incubator at 25°C. Middle growth culture was centrifuged and rinsed twice with 3 ml of 0.3% sucrose solution. Cells were resuspended with 300 μl of 0.5% sucrose solution, and the suspensions were divided in another 1.5 ml micro tube with 100 μl as competent cells. Approximately 1–5 μg of plasmid DNA solution was added to the competent cell solution, and electroporation was carried out under high-voltage conditions (25 kV/cm and 800 V with a 25-mF capacitor) with a Gene pulser (Bio-Rad, Richmond, CA).

**Inoculation assay.** *A. tumefaciens* strains were inoculated on germinated plant stems by wounding with a needle that was first dipped in a bacterial full-growth culture. After inoculation, the wounded site was covered with a damp cotton to prevent desiccation. The plants were grown continuously in the chamber and tumor formation on the inoculated site was observed 3–4 weeks after inoculation. At least 30 plants were inoculated with *A. tumefaciens*, and withered plants were excluded from final observation. There were no crown galls on the reference plants wounded without bacterial infection.

**Biotype determination and opine utilization.** Biotype determination of KAT23 was carried out according to the method of Kerr and Panagopoulos (1977). Tests were (i) 3-ketolactose production ability, (ii) growth ability on 2% NaCl, (iii) survivability in a high-temperature test, (iv) acid formation from meso-erythritol, melezitose, and ethanol, (v) alkali formation from sodium malonate, sodium L-tartrate, and propionate, and (vi) reaction in litmus milk. Growth ability on selective medium was determined using media IA and 2E\(^21\) instead of the selective medium of the Kerr and Panagopoulos method.

Carbon source limiting cultivation was performed with KAT23. Middle growth culture of KAT23 in 20 ml of Luria Broth was centrifuged, rinsed 3 times, and resuspended with 20 ml of AB minimal medium without a carbon source. One hundred μl of cell suspension was added to 20 ml of fresh AB minimal medium supplemented with 0.4 μmol of di-3-octopine or di-L-nopaline, and cultured with slow shaking at 25 °C for 64 h. Cells were cultured in AB minimal medium without a carbon source as a negative control and in AB minimal medium
supplemented with D-glucose as a positive control. Bacterial growth was monitored by measurements of OD₆₀₀ absorbance using a spectrophotometer 0, 20, 28, 40, and 64 h after inoculation.

**PCR.** All primers to be amplified from Ti plasmid were taken from the sequence (accession no., AJ237588) and designed using Genetyx software (Software Development, Tokyo), and oligonucleotides were synthesized by Hokkaido System Science (Sapporo, Japan). DNA of the right end of T-DNA (1.4 kb) was amplified with the nos primer (5′ TCG TGG GGT TGC TTC TAG TTC TCC 3′) and ocd primer (5′ ATT TGC GAT CGA GGA TTT TTC TTG GGT TCG CTC GTC TAG TTC 3′) set. This region contains the right border sequence, 1 kb of the upstream and 300 bp of the downstream. That corresponds to the C terminus of nopaline synthetase to the N terminus of ornithine cyclodeaminase (Fig. 1). DNA of the left end of T-DNA (1.3 kb) was amplified with accF primer (5′ GGA TAA CCT GAG CTT CGA CAT TG 3′) and acc2 primer (5′ CTA CGA TTT CTC CGC TCA ACT AG 3′) set. This region contains the left border sequence, 700 bp of the upstream and 600 bp of the downstream. The amplification reaction was carried out using approximately 100 ng of total DNA or 10 ng of purified plasmid DNA as template DNA on a thermal cycler under suitable conditions. The amplified DNA was purified by agarose gel electrophoresis and extraction using the QIAquick® Gel Extraction Kit (Qiagen Science, Germantown, MD).

**Sequence analysis.** The DNA amplified by PCR was cloned into pT7Blue vector and sequenced on both strands using primer T7primer or U-19mer primer (EMD Bioscience, San Diego, CA). DNA sequencing was performed with an ABI PRISM® 310NT Genetic Analyzer (Applied Biosystems, Foster City, CA) and analysis was done with Genetyx software.

**GUS staining.** Excised plants cells were soaked in 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-GlucA; Sigma-Aldrich, St. Louis, MO) solution and incubated at 37°C overnight. After incubation, the solution was decanted and gall tissues were rinsed with 70% ethanol twice and decolored in 95% ethanol at 37°C for 12 to 24 h.

**Results and Discussion**

**Host range determination**

One of the *A. tumefaciens*, isolated by Kaku et al., 1999, strain AT96-6, showed infectivity on various soybean cultivars (Peking, Harosoy, Enrei, and Suzuyutaka). We named this strain soybean super virulent strain KAT23. To determine the more detailed host range of KAT23, an inoculation assay was carried out on the soybean and common bean. The broad host-range strain C58 was used as a reference strain. Inoculation was done by a simple method of wounding by multiple needles with bacterial full-growth medium. Four weeks after inoculation, plants that formed crown galls were scored, and the results are displayed as percentages of tumor-forming plants (Fig. 2). KAT23 was available to infect seven soybean cultivars (Peking, Suzuyutaka, Fayette, Enrei, Mikawashima, Wasemidori, and Jack), six common bean cultivars (Legulus, Morocco, Serena, Kentucky Wonder, and Minidoka). In our observation, there was a tendency for KAT23 inoculated plants to form large tumors more efficiently and rapidly than was the case with C58. In case of soybean, sensitivity to *A. tumefaciens* was very low, and only Peking and Jack, and possibly Fayette, showed some sensitivity. Our data confirmed that KAT23 and C58 demonstrated higher virulence for those cultivars than other cultivars. KAT23 infected Peking and Jack at 100% and Fayette at 95.6%, though the infectivity of C58 was 60% for Peking, 61.5% Jack, and 56.4% Fayette. Furthermore, there were distinguishing differences among other soybean cultivars that C58 infected, with very low efficiency Suzuyutaka (26.4%) and Enrei (19.2%), but KAT23 infected Suzuyutaka at 62.5% and Enrei at 79.5%. Other soybean cultivars, WaseMidori and Mikawashima, and three common bean cultivars, Legulus, Morocco, and Kentucky Wonder also showed significant differences in sensitivity, and KAT23 caused crown gall tumors on these cultivars almost 2-fold higher than C58. In addition, KAT23 was able to form crown galls on some peanuts and field pea cultivars with higher efficiency than C58, even though KAT23 has been reported as null for peanuts (data not shown).

This striking high infectivity of KAT23 indicates the possibility of its availability as a novel *A. tumefaciens*
for soybean or other *Leguminosae* plant transformation. Especially in soybean, Suzuyutaka and Enrei are commercially important cultivars in Japan, but it is difficult to obtain transgenic plants. We are considering that the difficulty of obtaining transgenic plants is due to tolerance for *A. tumefaciens* infection. Thus KAT23 might be applicable to an alternative soybean transformation system with a wide host range.

**Characterization of KAT23 and its Ti plasmid**

The results of biotype determination are represented in Table 1. Although the survivability at high temperatures of KAT23 was higher than the results of Kerr and Panagopoulos, the results indicate that KAT23 is to be classified into biotype II.

Carbon source limiting cultivation revealed that KAT23 was able to grow in medium containing DL-nopaline or D-glucose as a single carbon source, but not DL-octopine (Fig. 3). Generally, opines are detected in plant crown gall tumors infected with wild-type *A. tumefaciens*.

**Table 1. Physiological and Biochemical Tests of A. tumefaciens KAT23**

<table>
<thead>
<tr>
<th>Test</th>
<th>Biotype I*</th>
<th>Biotype II*</th>
<th>Biotype III*</th>
<th>KAT23</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-keto-lactose production</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2% NaCl growth</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Max. growth temp.</td>
<td>37 °C</td>
<td>29 °C</td>
<td>35 °C</td>
<td>31 °C</td>
</tr>
<tr>
<td>Litmus milk</td>
<td>Alkali-Redn.</td>
<td>Acid</td>
<td>Alkali</td>
<td>Acid</td>
</tr>
<tr>
<td>Acid from <em>Meso</em>-erythritol</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Alcohol</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Melezitose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Alkali from Malonate</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>t-tartrate</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Propionate</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Selective medium</td>
<td>1A</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2E</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

*Kerr and Panagopoulos, 1977
**Brisbane and Kerr, 1983

**Fig. 2.** Inoculation Assay on Soybean and Common Bean.

Tumor formation (%) = (no. of plants that formed crown gall per no. of total plants inoculated with *A. tumefaciens* KAT23 and C58) × 100.

**Fig. 3.** Growth Curve of *A. tumefaciens* KAT23 Cultured in Medium Containing Opines.

The medium was supplemented with 20 mM of D-glucose (■), DL-octopine (○) or DL-nopaline (●) and without carbon source (□).
mefaciens. These opines’ synthesis abilities are provided by genes encoded on Ti plasmid, and the A. tumefaciens strain has the utilization abilities of the corresponding type of opine, and it too originated in Ti plasmid. Therefore we think that KAT23 has a nopaline catabolize pathway and thus might be encoded on nopaline-type Ti plasmid. To confirm this, we isolated the Ti plasmid from KAT23 and named it pTiKAT23. Using this pTiKAT23 DNA as a template DNA with the nos and ocd primer set, PCR was performed, and amplified 1.4-kb DNA was detected. These primers were designed from the consensus sequence of nopaline-type Ti plasmids. In addition, the left border sequence (TGG CAG GAT ATA TTG TGG TGT AAA C), which completely matched with nopaline type Ti plasmid (Fig. 4B). Right border sequence (A) and left border sequence (B) of T-DNA are aligned with typical border sequences. Octopine type borders A, B, C, and D have been published in DDBJ (accession no. AF242881). Nopaline type right and left borders contain pTiC58 (accession no. AJ237588), pTiSAKURA (accession no. AB016260), and pTiT37 (accession no. J01826, J01825). The nucleotide in the white square is a mismatched sequence with pTiKAT23.

**Fig. 4.** Nucleotide Sequence Alignment of T-DNA Borders.

These results indicate that KAT23 is a typical nopaline type strain, and this matches our expectation resulting from the opine utilization test. It is interesting that these partial sequence analyses indicate that pTiKAT23 is closely related with pTiC58 although KAT23 is classified in biotype II and C58 in biotype I. Although the virulent host range of A. tumefaciens is thought to be encoded mainly on Ti plasmid, it has been found that chromosomal genes are also involved in virulence.24–26 Perhaps the difference in chromosomal background between KAT23 and C58 might affect the host range.

**DNA transfer**

Binary vector pIG121-Hm, an RK2 derived plasmid, has the intron-containing β-glucuronidase (GUS) gene, kanamycin resistant gene, and hygromycin resistant gene in both border sequences.27) We inserted the green fluorescence protein (sGFP(S65T)) gene at the HindIII site between the β-glucuronidase gene and the kanamycin resistant gene (Fig. 5). This binary vector (pIGFP121-Hm) was introduced into KAT23 by electroporation and cultured in Luria Broth containing 50 mg/l of hygromycin. Full growth culture was inoculated on common bean (Minidoka) stems by the method with inoculation assay noted above. Following chamber culture, the common bean formed an enlarged tumor (Fig. 5A). Some cell clusters of the tumor exhibited green fluorescence under a fluorescence microscope (MZ FLIII, Leica Microsystems, Wetzlar, Germany) observation (Fig. 5B). Fluorescent sectors are shown with numbers 1 to 6 and size on a crown gall.

GUS staining of the excised tumor showed blue spots, indicating β-glucuronidase expression and accumulation, and some of them were in positions that yielded fluorescence (Fig. 5C). The β-glucuronidase gene contains an intron whereas crown gall tumors inoculated with KAT23 without any binary vector showed no GUS activities; hence these GUS activities did not originate from bacteria, but the plant genomic DNA acquired the β-glucuronidase gene. We obtained the same results for soybeans Peking, Suzuyutaka, and Enrei. They showed green fluorescence (Fig. 5E, G, and I), considerable sGFP(S65T) expression, and GUS activities (data not shown) in some crown gall tumors. This indicates that KAT23 was able to transfer T-DNA that was from both binary vector and pTiKAT23. This makes sense in that the border sequences of KAT23 was completely the same as nopaline type Ti plasmid and almost all the binary vectors harbored in the border sequences originated from nopaline type Ti plasmids. In addition, the overdrive sequence, an enhancer of border sequence recognition, of pTiKAT23 also completely matches other nopaline type Ti plasmid (data not shown). Although we have no insight into the key factor of the soybean super virulent ability of KAT23, we think that it is an important factor for the novel soybean transformation system whether the T-DNA transferring ability of KAT23 fully acts on a binary vector. Therefore, we think that KAT23 has the potential to function as a common A. tumefaciens-mediated plant transformation system using binary vector without any improvement in vectors or vir function. Our future experiments are

| A | Octopine type borderB | TGGCAGGATATAATCGGTTGTAATT |
| A | Octopine type borderD | TGGCAGGATATAATCGGTTGTAATT |
| B | Octopine type borderA | TGGCAGGATATAATCGGTTGTAATT |
| B | Octopine type borderC | TGGCAGGATATAATCGGTTGTAATT |
| pTiKAT23  | TGGCAGGATATAATCGGTTGTAATT |

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**Table 1.** Nucleotide Sequence Alignment of T-DNA Borders.
designed to construct an avirulent strain of KAT23, a T-DNA deletion mutant, and perform further analysis and discover application for plant transformation.

References


8) Zupan, J., Muth, T. R., Draper, O., and Zambrayski, P., The transfer of DNA from *Agrobacterium tumefaciens*...


