Production of the herb *Ruta chalepensis* L. expressing amyloid β-GFP fusion protein

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Abstract: The herb *Ruta chalepensis* L. exhibits medical effects, such as anti-inflammatory, central nervous system depressant, and antipyretic activities. However, a genetic transformation method has not yet been developed for this species. In this paper, a simple and efficient tissue culture and genetic transformation system for *R. chalepensis* is reported. An amyloid β-peptide (Aβ) gene, which is considered to be a causative agent of Alzheimer’s disease (AD), fused with green-fluorescent protein (GFP), was introduced into *R. chalepensis*. When the leaves of *R. chalepensis* expressing Aβ-GFP were administered orally to C57BL/6J mice, serum anti-Aβ antibody titers of several mice were elevated without the use of an adjuvant. These results indicated that an oral vaccine against AD using *R. chalepensis* may be feasible. *R. chalepensis* is rich in bioactive compounds that may have synergistic effects with the vaccine for AD. Plant-derived vaccines are safer and cheaper than those produced from animal cells or microbes, because plants can serve as biofactories at low cost and with high biosynthetic capacity.

Keywords: Alzheimer’s disease, amyloid β-peptide, *Ruta chalepensis* L, vaccine

Introduction

Alzheimer’s disease (AD) is a neurodegenerative disorder affecting more than 35 million people worldwide. Cerebral amyloid plaques and prominent neurofibrillary tangles (NFTs) in the medial temporal lobe are important pathological features of AD.1,2) The plaques are largely composed of amyloid β-peptide (Aβ) peptides consisting of 37 to 43 amino acids, which are derived from the large amyloid precursor protein (APP), whereas NFTs are composed of microtubule-associated Tau protein. Rare autosomal dominant mutations in three genes, APP, presenilin 1, and presenilin 2, cause familial AD, which accounts for 2–3% of all AD cases. These proteins are substrates and enzymes of the Aβ-forming pathology.

In 1999, a novel vaccination therapy for Aβ was developed against AD.3) The experiments employed APP transgenic mice (PDAPP) that overexpressed mutant human APP and progressively developed plaques in an age-dependent manner. In the study, the transgenic mice were immunized with Aβ42 either before or after the onset of senile plaques. The treatment markedly reduced the progression of AD-like neuropathology. In 2001, the Elan Corporation examined the clinical application of Aβ immunization. However, during a phase II trial, 6% of the patients presented with acute meningitis as a side effect after the second immunization, and the clinical trial was suspended.4) Adjuvant-containing QS21 saponin had high immunological activity and induced cell-mediated immune responses and Aβ-reactive killer T cells that invaded the brain, causing allergic meningitis. However, a case report suggested that the vaccine was effective for Aβ clearance.5)

Many vaccination studies for AD have been conducted since then. Among the strategies, oral vaccination using an edible plant as a vehicle confers a number of benefits: 1) only the target protein or fragments are expressed, 2) purification of the target protein and cold storage are not required, and 3) the
cost is very low. We succeeded in stably expressing green-fluorescent protein (GFP)-conjugated Aβ in green pepper and brown rice. Oral vaccination of Tg2576 transgenic mice resulted in an elevation of Aβ antibody titer without any side effects, and a reduction of Aβ in the immunized mice.

The immunological effect of vaccines relying on oral–intestinal mucosal immunization tends to be weak, and this method induces immunological tolerance. To prevent this tolerance, specific adjuvants can be used. Cholera toxin B subunit (CTB) is frequently used as the oral adjuvant, and it effectively induces an immunological reaction against Aβ. CTB is considered safe; however, application of CTB to humans remains prohibited. A safer oral adjuvant to raise the antibody titer against Aβ is needed.

The herb Ruta chalepensis L., commonly known as “fringed rue,” is native to the Mediterranean region. It was introduced to various countries and can also be found on the main island of Okinawa, Japan. This plant is a rich source of important secondary metabolites, such as alkaloids, flavonoids, coumarins, and saponins, and it has been renowned since ancient times for its medical uses. On the Ryukyu islands, the herb is called “Isha-Nakashi Kusa,” which translates to “grass that makes doctors cry.” We believe that a genetically modified R. chalepensis can offer greater benefits to humans compared with the wild type. However, to the best of our knowledge, a genetic transformation method for R. chalepensis has not yet been established.

Aβ has been expressed in edible plants (green pepper, rice, and potato), but these plants generally have few bioactive compounds. Herbs contain various secondary metabolites that may have synergistic effects with the vaccine. In this study, we used R. chalepensis as the host plant for an oral AD vaccine, because R. chalepensis could have synergistic effects with the vaccine, such as anti-inflammatory activity and various effects on the central nervous system.

We first established a tissue culture method for R. chalepensis and then introduced Aβ into the herb. Furthermore, we examined the possibility of an R. chalepensis-derived AD vaccine using mice.

Materials and methods

Plant materials. R. chalepensis that grew naturally on the main island of Okinawa, Japan was used for the experiments. The surfaces of the leaves and stems were sterilized in 70% ethanol for 3 s and then in 0.5% sodium hypochlorite for 15 min, followed by rinsing four times in sterile distilled water.

Culture conditions for shoot and root organogenesis from explants. To establish culture conditions, transverse sections with a thickness of 1 mm were cut from the leaves and stems. For shoot organogenesis, the explants were placed on Murashige and Skoog (MS) medium with a combination of different concentrations of 1-naphthaleneacetic acid (NAA) and thidiazuron (TDZ) and with 1% Bacto agar. For rooting, the cultures were placed on MS medium with different concentrations of 3-indoleacetic acid (IAA) and 1% Bacto agar. The cultures were incubated at 25 °C in a 16-h photoperiod using cool white fluorescent lights at 40 µmol m⁻² s⁻¹.

Transformation. The human Aβ42 gene fused with a green GFP gene was introduced into R. chalepensis according to the method detailed below.

The transformant LBA4404 [pIG121-Hm (sGFP + Aβ)] was used for Agrobacterium-mediated transformation. The bacteria were grown overnight on a lysogeny broth (LB) medium with 50 mg/L of kanamycin, 50 mg/L of hygromycin and 1.2% Bacto agar at 25 °C. The bacteria were then suspended in a plant infection medium [90% (v/v) MS medium and 10% (v/v) LB broth, 50 µM acetosyringone]. The leaves or stems of R. chalepensis were cut into 1-mm transverse sections and immersed in the medium containing the bacteria for 15 min.

The leaves or stems were transferred to a cocultivation medium [MS medium containing 1 µM NAA and 1 µM benzylaminopurine (BAP) modified to pH 5.2 with 50 µM acetosyringone] and incubated in the dark at 22 °C for 3 days. After co-cultivation, the leaves or stems were washed with medium (MS medium, containing 400 mg/L carbenicillin, pH 5.8) to eliminate bacteria.

The washed leaves or stems were cultured on a selective medium (MS medium containing 1 µM NAA and 1 µM BAP with 15 mg/L hygromycin B and 300 mg/L carbenicillin) at 25 °C under a 16-h photoperiod.

Plantlets directly regenerated on the cut surface of the leaves and stems and were transferred onto MS medium containing 15 mg/L hygromycin B and 200 mg/L carbenicillin. These plantlets were incubated at 25 °C in a 16-h photoperiod, and fully grown plantlets were planted in soil.
Southern blotting analysis. Extracted leaf DNA was digested with XhoI. Electrophoresis was conducted with 0.8% agarose gel, and the DNA was blotted onto a nylon membrane (Hybond-XL; GE Healthcare, U.S.A.) and subjected to Southern hybridization for 4 h at 65 °C. A GFP probe (764 bp) with [32P] dCTP was used during this process. Labeling of the probe was performed using the Megaprime DNA labeling system (GE Healthcare).

Western blotting analysis. Freeze-dried leaves (40 mg) were crushed while frozen, and total protein was extracted for 1 h with 400 µL protein extraction buffer [450 mM Tris-HCl (pH 8.45), 12% glycerol, 4% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol]. Five microliters of each sample was used for Tris-tricine-SDS polyacrylamide gel electrophoresis (SDS-PAGE) (12% T, 3.3% C) together with 5, 10, 20, and 40 ng of Aβ (human, 1–42) (Peptide Institute, Inc., Japan) as a standard, and the samples were transferred to a Hybond-P PVDF membrane (GE Healthcare) using an electroblotting system (AE-6677; ATTO Corp., Japan). The membrane was incubated in blocking buffer (5% skim milk, T-PBS) and treated for 1 h with β-amyloid, 1–16 (6E10) monoclonal antibody [Signet (Covance Inc., U.S.A.)], at 25 °C. The GFP-Aβ fusion protein bound to the membrane was detected using anti-mouse horseradish peroxidase conjugate electrochemiluminescence (ECL) plus a Western blotting reagent pack (GE Healthcare) and an ECL plus Western blotting detection system (GE Healthcare).

Mouse immunization. Fresh leaves were crushed while frozen and mixed in distilled water. The quantity of leaf matter administered to each mouse was adjusted to deliver 0.2 µg of Aβ, and it was mixed with or without CTB (List Biological Laboratories, U.S.A.) (1 µg per mouse).

C57BL/6J male mice (Charles River, Japan) were divided into three groups (six mice per group) and fed orally with a feeding needle (only R. chalepensis, Aβ-containing R. chalepensis, or Aβ-containing R. chalepensis + CTB). The mice were administered doses once per week from 8 to 11 weeks of age. As a booster, 2 µg of chemically synthesized human Aβ mixed with Freund’s incomplete adjuvant was injected into all mice at 14 weeks of age. Blood serum was collected at 8, 12, 14, and 16 weeks of age. The mice were maintained at 25 °C with a 12-h light/dark cycle. The anti-Aβ antibody titer of the blood serum was measured in accordance with the method of Yoshida et al. (2011).13)

Results

To establish culture conditions for efficient regeneration of R. chalepensis, leaf and stem explants were placed on media with a combination of different concentrations of NAA (0, 1, and 10 µM) and TDZ (0, 1, 10, 30, and 100 µM) (Table 1, Fig. 1). TDZ was required for shoot organogenesis in both leaf and stem explants. The TDZ concentrations did not affect the efficiency of shoot organogenesis within 1–100 µM TDZ (Table 1). However, if TDZ concentrations were high, the growth stage of the regenerated shoots remained at the shoot primordia stage, or as tiny multiple shoots. NAA additionally affected shoot organogenesis. In leaf explants, the efficiency of shoot organogenesis was clearly lower in 0 µM NAA than in 1 or 10 µM NAA. In stem explants, the efficiency of shoot organogenesis was not lower in 0 µM NAA than in 1 or 10 µM NAA, and the growth stage of the shoot remained at the shoot primordia stage in 10 µM NAA. Growth was also examined (Table 2). The fresh weight of explants was measured at 8 weeks of culture. For leaf explants, the explants grew larger in a combination of NAA (1 and 10 µM) and TDZ (10, 30, and 100 µM) than in other combinations with NAA. For stem explants, explants grew larger with 10, 30, and 100 µM TDZ. IAA was not required for rooting from plantlets (Table 3).

We used regeneration medium with 1 µM NAA and 1 µM TDZ for shoot regeneration, and hormone-free rooting medium for the transformation protocol.
Plantlets were regenerated from both leaves and stems. Southern blotting analysis was used to confirm the transformation in individual plants. The introduction of the Aβ-GFP gene into *R. chalepensis* was confirmed by Southern blotting analysis (Fig. 2).

Western blotting was used to investigate the accumulation of Aβ-GFP fusion protein in the leaves of genetically modified *R. chalepensis* (Fig. 3). A band of approximately 32 kDa, which corresponded to the mobility of the GFP + Aβ42 (31.5 kDa), was detected by SDS-PAGE. The signal intensity of the band was compared with that of Aβ42 as a control, and differences in Aβ concentration were observed among individuals. The highest concentration of Aβ, which was less than 10 µg/g (5 ng/0.5 mg), was found in freeze-dried leaves of line 14.

The immunogenicity of genetically modified *R. chalepensis* was examined by feeding the leaves to male C57BL/6J mice aged from 8 to 11 weeks (Fig. 4). After immunization, anti-Aβ antibody titers

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**Table 2. Effect of thidiazuron (TDZ) and 1-naphthaleneacetic acid (NAA) on shoot growth (fresh weight) from leaf (upper value) and stem (lower value) explants**

| TDZ concentration (µM) | 0  | 1  | 10 | 30  | 100 |
|------------------------|----|----|----|-----|-----|
| **NAA concentration (µM)** | 0  | No growth | 74 ± 59 | 101 ± 104 | 81 ± 96 | 49 ± 52 |
|                         | 1  | No growth | 244 ± 123 | 467 ± 154 | 547 ± 296 | 424 ± 287 |
|                         | 10 | No growth | 159 ± 107 | 302 ± 174 | 316 ± 226 | 377 ± 182 |
|                         | 1  | No growth | 215 ± 122 | 228 ± 75 | 140 ± 69 | 239 ± 107 |
|                         | 10 | No growth | 118 ± 38 | 375 ± 212 | 426 ± 157 | 363 ± 140 |

Ten transverse 1-mm sections were used for each combination of TDZ and NAA. Value indicates fresh weigh (mg)/explant + SD at 8 weeks of culture.
were increased in several mice fed A\(\beta\)-containing leaves with or without CTB. In contrast, an increase in the antibody titer was not observed in mice fed wild-type leaves.

**Discussion**

Vaccines targeting the molecule associated with chronic noncommunicable diseases, such as AD and Parkinson’s disease, are promising strategies that may lead to new therapies.\(^{18,19}\) Immunotherapy against AD is focused mainly on A\(\beta\), because A\(\beta\) accumulation is the first step toward chronic degeneration of the brain. However, anti-A\(\beta\) monoclonal antibodies have shown side effects such as vasogenic edema, representing a significant concern with respect to the safety of passive immunotherapies.\(^{20}\) In addition, active immunotherapy using preaggregate A\(\beta\) and saponin adjuvant (AN1792) was abandoned because of the development of meningitis.\(^{4}\)

We first demonstrated that the induction of adverse humoral responses was due to injection of the antigen, and oral administration of the A\(\beta\) antigen showed no serious adverse effects.\(^{10}\) In the human body, immunological reactivity against “the self” is usually suppressed. This phenomenon is called immunotolerance. Self-immunotolerance is affected by the balance between Th1 and Th2 helper T cells.\(^{15}\) When the balance between these helper cells and Th1 cells is disturbed, cellular reactions to the self increase, tending to give rise to autoimmune responses such as meningitis. When Th2 cells are activated, antibody production is induced and anti-inflammatory cytokines are released, alleviating the inflammation induced by Th1 cells. Mucosal immunization induces Th2 cells and is thought to be a safer vaccine therapy. We reported previously that mice were able to produce antibodies against A\(\beta\) following oral immunization and the induction of immunotolerance to vehicle proteins.\(^{11}\) Therefore, the side effects induced by oral adjuvant can be avoided for safer future vaccine therapies.

Active immunization also offers several advantages, such as lower costs and a lower number of
doses in comparison to passive immunization. Developing safe and effective vaccines requires omitting proinflammatory adjuvants, but without adjuvant to elicit therapeutic levels of anti-Aβ antibodies, this has proven difficult.

The TDZ and NAA used in experiments for shoot regeneration are plant hormones. TDZ has an effect similar to that of cytokinins, which are considered to participate in differentiation, especially shoot organogenesis. Lièvre et al. examined shoot regeneration in a closely related species, *R. graveolens*. They examined the effect of BAP, a kind of cytokinin, at 0, 0.44, 4.44, or 8.89 µM (0, 0.1, 1, or 2 mg/L), and a concentration of 4.44 µM had a higher differentiation effect than at 0.44 µM and 8.89 µM. In this study, shoots were generated at a low concentration (1 µM) of TDZ. NAA is an auxin participating in growth and root organogenesis, and NAA may promote shoot regeneration by combining with cytokinin. Lièvre et al. did not examine the effect of NAA. We showed that the addition of NAA was effective for differentiation of *R. chalepensis*. Because higher concentrations of plant hormones may have side effects, we decided to use hormones at low concentrations (1 µM TDZ and 1 µM NAA) for the production of genetically modified *R. chalepensis*. As a result, we showed that genetically modified *R. chalepensis* could be produced effectively with low concentrations of TDZ and NAA. However, there is a possibility that more-efficient genetic modification can be achieved with lower concentrations, less than 1 µM, of these plant hormones.

In the genus *Ruta*, an *Agrobacterium*-mediated transformation was successful in *R. graveolens*. *Ruta*, commonly known as an herb rue, contains many secondary metabolites, and plants in this genus have been used in herbal remedies. *R. chalepensis*, used in the current study, is also a well-known species in this genus, and to the best of our knowledge, this is the first time that a foreign gene has been expressed in *R. chalepensis*. Genetic modification is able to produce a more useful *Ruta* phenotype than the wild type, *i.e.*, genetically modified *Ruta* can modify metabolic pathways for producing novel secondary metabolites, and can additionally produce new peptides for medical use. In the current study, we introduced Aβ into *R. chalepensis* to produce an oral vaccine against AD.

GFP is used for the selection of genetically modified plants. We also used GFP as an enhancer of Aβ titer. GFP is larger than Aβ; therefore, the titer for GFP may increase further. GFP is thought to be non-toxic in humans, and previous experiments have suggested that GFP does not have any side effects in mice. However, the effects of oral administration of GFP in humans should be determined.

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**Fig. 4.** Titers of antibodies against Aβ in serum before and after immunization. Anti-Aβ antibody titers for each mouse are shown. Horizontal lines show the average.
Aβ was administered orally to mice over a short period (3 weeks), and two of six mice (Aβ alone) and one of six mice (Aβ + adjuvant, CTB) produced anti-human-Aβ antibodies. Regarding clinical applications, we are considering long-term administration to patients who have not yet developed AD or are in the early stage of onset. In such cases, marked effects may be expected. Consequently, a long-term study in mice is under preparation.

Oral vaccinations using plant-derived antigens have fewer side effects than those administered by injection. In addition to the benefit of immunological safety, plant-derived vaccines are considered to be safer and cheaper than those produced from animal cells or microbes. A vaccine produced from animal cells or microbes requires refinement during the manufacturing process, because animal cells may contain viruses and prions that might infect humans. In addition, microbes may contain endotoxins. In contrast, plant-derived vaccines can be administered directly to people. Therefore, a plant-derived vaccine can be produced relatively cheaply.

When Aβ-containing plants (green pepper, rice, and potato) were administered to mice, their serum anti-Aβ antibody titers were elevated. In these experiments, CTB was added as an adjuvant. In the current study, Aβ-containing R. chalepensis could elevate serum anti-Aβ antibody titers without the addition of CTB (Fig. 4). Crude saponins have been reported to improve the immune response to the addition of CTB (Fig. 4). Crude saponins have been reported to improve the immune response to the addition of CTB (Fig. 4). Crude saponins have been reported to improve the immune response to the addition of CTB (Fig. 4).

R. chalepensis may contain bioactive components that stimulate the immune system of mice. It has been suggested that chronic inflammation triggers AD. Because R. chalepensis has anti-inflammatory effects, the unidentified bioactive components of R. chalepensis are considered to provide some benefits to the central nervous system, including sedative, hypnotic, anxiolytic, anticonvulsant, and antinociceptive effects. Therefore, the bioactive compounds of R. chalepensis may have synergistic effects with the vaccine for AD. Based on these results, Aβ-containing R. chalepensis may be suitable for administration as an oral medication to patients with AD.

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