

Expression of a Major House Dust Mite Allergen Gene from *Dermatophagoides farinae* in *Lotus japonicus* Accession Miyakojima MG-20

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Received 13 September 2004/Accepted 17 November 2004

Transformation of a model legume *Lotus japonicus* accession Miyakojima MG-20 was examined using *Agrobacterium tumefaciens* with a binary expression vector. Using the improved transformation method, we introduced a major allergen gene from a house dust mite, *Der f 1*, into MG-20. Analyses by Southern hybridization, reverse transcription (RT)-PCR, and Western blotting showed that the *Der f 1* gene was integrated into the genome of *L. japonicus*, expressing the gene product in the T1 lines. Our results imply future application of oral allergen-specific immunotherapy using legume plants.

[Key words: *Lotus japonicus* accession Miyakojima MG-20, *Agrobacterium tumefaciens*, pIG121Hm, *Dermatophagoides farinae*, *Der f 1*, oral allergen-specific immunotherapy]

Legume plants are agronomically widely cultivated, for crops, pastures, and manures. To improve their potential value as a commercial product, genetic modification can be used to confer new characteristics. The use of such plants as food vaccine is one strategy currently being considered. However, most important legume species, e.g., soybean and pea, are difficult to transform. Therefore, we need model legumes for which the transformation methods are established. *Lotus japonicus* and *Medicago truncatula* are widely used in genetic and molecular studies as model legumes (1, 2). In the case of *L. japonicus*, accession Gifu B-129 is used for transgenic studies (1, 3, 4). However, B-129 requires intense light to produce seeds, so that indoor handling is difficult in terms of generating transgenic plants. Kawaguchi reported on an early-flowering *L. japonicus* accession Miyakojima MG-20, which produces seeds for two months under moderate light conditions (5). The life cycle of MG-20 is roughly one month shorter than that of B-129. Unfortunately, the transformation methods developed for B-129 are not considered as optimal for MG-20. In this paper, we describe a transformation method suitable for MG-20, modified from original methods for B-129. By using this improved method, we attempted to introduce a major allergen gene *Der f 1* from a house dust mite *Dermatophagoides farinae* (6) into MG-20. *Der f 1* protein is detected in mite feces (7, 8), which has been recognized clinically as one of the most critical allergens. It has been confirmed that a purified *Der f 1* protein expressed in *Esch-*

erichia coli is bound by IgE from allergenic patients, so the protein has the potential to work well for immunotherapy (9). Here, we have successfully expressed the *Der f 1* gene and detected *Der f 1* protein in transgenic MG-20 plants.

MATERIALS AND METHODS

Plant materials and plant growth conditions *L. japonicus* accession Miyakojima MG-20 was used. Seeds were scarified by gentle treatment with sand paper and sterilized (8 min in 1% sodium hypochlorite), washed five times in sterilized water, and immersed overnight at room temperature. The seeds were then germinated on 1% Bacto Agar plates, put in the growth cabinet for 3 d at 23°C under dark conditions, followed by 2 d at a 23°C 16-h light/20°C 8-h dark cycle.

Agrobacterium strain and vector construct *Agrobacterium tumefaciens* LBA4404 (10) was used for the transformation of *L. japonicus*. The *Der f 1* gene was amplified by the polymerase chain reaction (PCR) from the plasmid pBSDerf1 (T. Aki, Ph.D. thesis, Hiroshima Univ., 1994) using the Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). The oligonucleotide primer used for PCR was as follows: 5'-TGAAATTCGTT TTGGCCATT-3' as a sense primer and 5'-GTTTCACATGATTACA ACATA-3' as an antisense primer. The PCR products were separated by agarose gel electrophoresis and a 1-kb fragment corresponding to the *Der f 1* gene was extracted using the GeneClean III kit (Qbiogene, Quebec, Canada). The extracted fragment was cloned into the multicloning site of the pGEM T-easy vector. A 1-kb *Xba*I-*Sac*I fragment containing the *Der f 1* gene was inserted into pIG121Hm (11), which was digested with the same restriction enzymes to delete the intron-GUS gene and the resultant plasmid was named pDerf1 (Fig. 1). The pDerf1 plasmid was transferred into *A. tumefaciens* LBA4404 by an electroporation method at 2.5 kV and 129 Ω for 5 ms using a BTX 600 Electro Cell Manipulator

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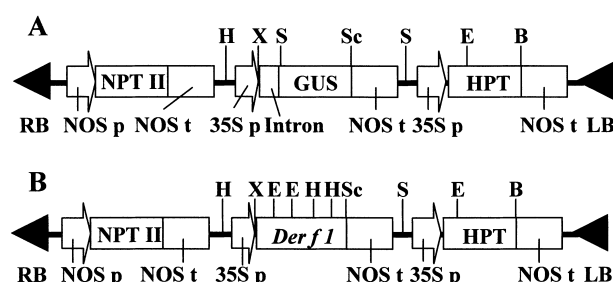


FIG. 1. Structure of T-DNA region in plG121Hm (A) and pDerf1 (B). RB, Right border; LB, left border; NPT II, neomycin phosphotransferase; GUS, β-glucuronidase; HPT, hygromycin phosphotransferase; NOS p, neomycin phosphotransferase promoter; NOS t, 3' signal of neomycin phosphotransferase; 35S p, 35S promoter; H, HindIII; X, XbaI; S, SalI; Sc, SacI; E, EcoRI; B, BamHI.

(BTX Inc., San Diego, CA, USA). *A. tumefaciens* transformants were selected on Luria-Bertani (LB) medium containing 100 μg/ml rifampicin and 50 μg/ml kanamycin.

Plant regeneration conditions All regeneration media were based on Gamborg's B5 medium (12). Callus induction medium (CIM) contained 10 mM (NH₄)₂SO₄, 2% sucrose, 0.3% phytagel (Sigma, Taufkirchen, Germany), α-naphthalene acetic acid (NAA), 6-benzyl amino purine (BAP), 300 μg/ml Claforan (Aventis Pharma, Strasbourg, France), and 25 μg/ml geneticin (Sigma). Shoot induction medium (SIM) contained 10 mM (NH₄)₂SO₄, 2% sucrose, 0.3% phytagel, NAA, and BAP. Shoot elongation medium (SEM) contained 10 mM (NH₄)₂SO₄, 2% sucrose, 0.3% phytagel and BAP. Root induction medium (RIM) contained 1% sucrose, 0.4% phytagel and NAA. Root elongation medium (REM) contained 1% sucrose and 0.4% phytagel. The media contained full (CIM, SIM, SEM) or half (RIM, REM) strength Gamborg's B5 Medium Salts Mixture (Wako Pure Chemical Industries, Osaka), and B5 vitamins (Sigma). One hundred hypocotyl segments were examined for callus regeneration and formation of adventitious shoots at various concentrations of NAA (0–0.1 μg/ml) and BAP (0–1.0 μg/ml) as plant growth regulators. *A. tumefaciens* was grown on LB solid medium containing 100 μg/ml rifampicin for 2 d at 28°C. *A. tumefaciens* was scraped off and resuspended in YMB liquid medium (13) for co-cultivation. Hypocotyls were cut on a two-layer sterile filter paper stack that was soaked with *A. tumefaciens* suspended in YMB medium. A stack of four layers of sterile filter paper was soaked with the co-cultivation medium (3) containing 20 μg/ml acetosyringone (Sigma) for co-cultivation. The hypocotyl explants were transferred to the co-cultivation plates, and incubated for 6 d at 23°C under dark conditions. The top filter paper with explants was transferred to CIM, and incubated for 2–5 weeks at a 23°C 16-h light/20°C 8-h dark cycle. Shoot induction and shoot elongation were carried out on the SIM and SEM, respectively, for 3–6 weeks under the same conditions. Explants and shoots were transferred to fresh media every week. The elongated shoot was cut and put onto RIM, and incubated for a week under the same conditions. Root elongation was performed on REM, and plants were incubated for 3–4 weeks under the same conditions.

Genomic DNA isolation and Southern hybridization analysis Plant genomic DNA was prepared from transformants and the wild type according to the cetyltrimethylammonium bromide method (14). Genomic DNA samples (7 μg) were digested with *Xba*I and a 1-kb *Derf1* fragment was used as a probe and a positive control. Digested DNA samples were electrophoresed on a 0.8% TAE agarose gel and transferred to Hybond N⁺ nylon membrane (Amersham Biosciences, Uppsala, Sweden) by standard methods (15). Labeling, hybridization, and washing were performed according to the instruction manual of the AlkPhos Direct

Nucleic Acid Labeling and Detection Systems (Amersham Biosciences).

Reverse transcription polymerase chain reaction (RT-PCR) analysis Plant total RNA samples were prepared from transformants and the wild type using the RNeasy Mini kit (Qiagen, Basel, Switzerland). RT-PCR analysis was performed following the RACE protocol (16) with some modifications. The total RNA samples were mixed with 50 μM anchor primer oligo dT mixture, 10 mM DTT, 0.2 mM dNTP, first-strand buffer, and 20 U of RNase inhibitor (Promega, Basel, Switzerland) in RNase-free water to a final volume of 20 μl. The mixture was heated at 65°C for 10 min and cooled on ice rapidly. After addition of 40 U of SuperScriptII (Invitrogen, Carlsbad, CA, USA), cDNA synthesis was carried out at 50°C for 30 min. The RTase was denatured by heating the sample at 75°C for 15 min. The cDNA samples were used for PCR with a set of specific primers at a final concentration of 1 μM, as described previously, 1.25 U ExTaq polymerase, 0.1 mM dNTP, and ExTaq polymerase buffer. The amplification was carried out as follows: 1 min at 94°C; 1 min at 55°C; 1 min at 72°C (30 cycles).

Western blotting analysis Total soluble protein was extracted from the leaves of transformed plants and the wild type by grinding them with a pre-cooled mortar and pestle in liquid nitrogen. Three hundred μl of Tris-HCl buffer (pH 7.7) per 100 mg of leaves were added and the homogenate was centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant containing total soluble protein was used for further analysis. Protein concentrations were determined by the Bradford Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Extracted protein was subjected to 12% SDS-PAGE using pre-stained molecular weight markers, and separated proteins were transferred to a nitrocellulose membrane at a constant current of 120 mA overnight. Blocking was carried out using 5% skim milk powder in TTBS (Tween Tris-buffered saline) for 1 h. For probing the blot with antibody, the membrane was incubated with mouse anti-*Derf1* monoclonal antibody (Seikagaku Kogyo, Tokyo) at a 1:100 dilution. The blot was probed with alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) (Promega) (1:4000) for 30 min. Bound antibodies were detected by BCIP and NBT (Promega).

RESULTS AND DISCUSSION

We have determined hormone conditions for the shoot regeneration of *L. japonicus* accession Miyakojima MG-20 because such conditions are crucial for efficient transformation. Various concentrations of NAA (from 0 to 0.1 μg/ml) and BAP (from 0 to 1.0 μg/ml) were tested (Table 1). Although callus induction was observed efficiently in all tested concentration combinations, except when no NAA or BAP was used, the efficiency of shoot induction varied considerably depending on the concentration of hormones. The highest rate of shoot induction (96%) was obtained with a combination of 0.05 μg/ml NAA and 0.2 μg/ml BAP. With the combination of 0.05 μg/ml NAA and 0.5 μg/ml BAP that was used for B-129, shoot induction decreased to 62% in the case of MG-20. As a result, we set the optimum condition for regeneration of MG-20 as a combination of 0.05 μg/ml NAA and 0.2 μg/ml BAP. Aside from the fact that MG-20 has a shorter life cycle than B-129 (5), these optimized hormone concentrations for shoot regeneration can shorten the whole transformation procedure from preparation of hypocotyls for infection to establishing stable homozygous transgenic lines in T2 seeds by about three months. Therefore, we conclude that MG-20 is suitable for assess-

TABLE 1. Efficiencies of callus formation and shoot induction

BAP ($\mu\text{g/ml}$)	Explants forming geneticin ^R calli (%)					Explants forming shoots (%)				
	NAA ($\mu\text{g/ml}$)					NAA ($\mu\text{g/ml}$)				
	0	0.01	0.02	0.05	1.0	0	0.01	0.02	0.05	1.0
0	0	0	0	0	0	0	0	0	0	0
0.1	0	100	100	100	100	0	6	30	28	40
0.2	0	100	100	100	100	0	4	42	96	68
0.5	0	100	100	100	100	0	16	18	62	28
1.0	0	100	100	100	100	0	2	34	24	24

NAA, α -Naphthaleneacetic acid; BAP, benzylaminopurine.

Results represent the average of two independent experiments. Total number of explants used in this experiments was 100.

ment of heterologous expression systems as an efficient host for transformation, when one needs to evaluate the potential property of a target gene introduced into crop legumes.

Using this improved method, we have transformed the *Der f 1* gene into MG-20 (Fig. 1). In order to detect the transgene in the host genome, we analyzed candidate transgenic T0 plants by Southern hybridization (Fig. 2A). Using *Xba*I to digest genomic DNA, the probe sequence including the *Der f 1* coding region could detect one integration event per signal. After screening of T0 candidates by PCR (data not shown), almost all of the candidate lines showed positive signals for the *Der f 1* gene. The copy number of the *Der f 1* gene varied depending on the transgenic lines, from

single to multiple copies. Usually, transgenic lines with fewer inserts are favorable because multiple inserts may cause interference of expression, resulting in low expression of the transgene. We harvested T1 seeds obtained from T0 lines that were indicated by Southern hybridization to have one or two copies of the gene. Similarly, several T1 lines from identical T0 parents were analyzed by PCR (data not shown) and Southern hybridization. As a result, one or two signals were detected with the *Der f 1* gene probe (Fig. 2B). In some lines, two signals were detected equally in siblings (e.g., Fig. 2B, lanes 1 and 2), indicating that two independent integration events occurred within a tightly linked region. To confirm whether the *Der f 1* gene was transcribed, total RNA was extracted from the leaves of T1 lines. *Der f 1* mRNA was detected in total RNA preparations by RT-PCR (Fig. 3A). Direct PCR amplification of total RNA preparations without RTase showed no fragment (Fig. 3A, lanes 4 to 6), indicating that the amplification product was not due to DNA contamination in the total RNA samples. A non-transformed plant did not show the presence of *Der f 1* mRNA (data not shown). Moreover, we analyzed the *Der f 1* gene products from T1 leaves. Crude soluble proteins were analyzed by Western blotting using a mouse anti-*Der f 1* monoclonal antibody. Transformed lines that synthesized *Der f 1* protein were identified in all T1 lines analyzed (Fig. 3B). The molecular size of the plant-synthesized *Der f 1* protein was found to be approximately 25 kDa, corresponding to the size of native *Der f 1* purified from *D. fariniae*. This result suggests that the *Der f 1* protein is not modified

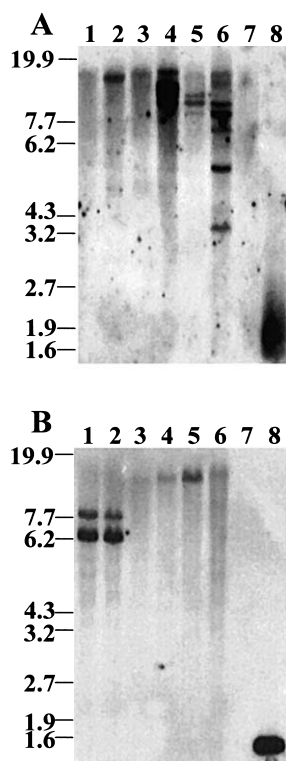


FIG. 2. Analysis of integrated DNA in T0 and T1 plants. (A) Detection of the *Der f 1* gene in T0 transgenic plants by Southern hybridization. Lane 1, Genomic DNA from 6-1; lane 2, 26-2; lane 3, 12-2; lane 4, 5-1; lane 5, 10-4; lane 6, 2-2; lane 7, non-transformed plant; lane 8, *Der f 1* fragment. (B) Detection of the *Der f 1* gene in T1 transgenic plants by Southern hybridization. Lane 1, 12-2-5; lane 2, 12-2-7; lane 3, 26-2-1; lane 4, 26-2-7; lane 5, 26-2-9; lane 6, 6-1-5; lane 7, non-transformed plant; lane 8, *Der f 1* fragment.

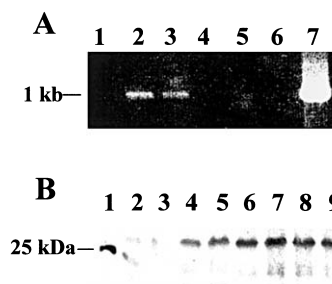


FIG. 3. Expression analysis in T1 plants. (A) Detection of the *Der f 1* mRNA in T1 transgenic plants using RT-PCR. Lanes 1 and 4, cDNA from a non-transformed plant; lanes 2 and 5, 12-2-1; lanes 3 and 6, 26-2-9; lane 7, *Der f 1* fragment, with RTase (lanes 1-3) or without RTase (lanes 4-6). (B) Detection of the *Der f 1* protein in T1 transgenic plants using Western blot analysis. Lane 1, Proteins from a *Der f 1* purified from mite; lanes 2 and 3, non-transformed plant; lanes 4 and 5, 12-2-1; lanes 6 and 7, 26-2-9; lanes 8 and 9, 6-1-5.

(e.g., by glycosylation) in this model legume, indicating that the putative IgE epitope is conserved.

In conclusion, we have succeeded in producing Der f 1 protein in the model legume *L. japonicus* accession Miyakojima MG-20. Recently, allergen-specific immunotherapy (AIT) has become the focus of public attention (17). The expression of Der f 1 in legume plants represents a useful tool in establishing a safer and more effective method of oral AIT. In order to focus on the application of our results to AIT, a seed-specific promoter from, for example, the soybean conglycinin gene (18) would need to be applied to a suitable expression system in legume plants.

ACKNOWLEDGMENTS

This research was supported by the 21st Century Center of Excellence Program of the Ministry of Education, Culture, Sports, Science and Technology, Japan. We thank K. Nakamura for providing us with pIG121Hm.

REFERENCES

1. Handberg, K. and Stougaard, J.: *Lotus japonicus*, an autogamous, diploid legume species for classical and molecular genetics. *Plant J.*, **2**, 487–496 (1992).
2. Barker, D. G., Bianchi, S., Blondon, F., Dattee, Y., Duc, G., Essad, S., Flament, P., Gallusci, P., Génier, G., Guy, P., Muel, X., Tourneur, J., Dénarié, J., and Huguet, T.: *Medicago truncatula*, a model plant for studying the molecular genetics of the *Rhizobium*–legume symbiosis. *Plant Mol. Biol. Rep.*, **8**, 40–49 (1990).
3. Stiller, J., Martirani, L., Tuppale, S., Chian, R. J., Chiurazzi, M., and Gresshoff, P. M.: High efficiency transformation and regeneration of transgenic plants in the model legume *Lotus japonicus*. *J. Exp. Bot.*, **48**, 1357–1365 (1997).
4. Lombari, P., Ercolano, E., El-Alaoui, H., and Chiurazzi, M.: A new transformation-regeneration procedure in the model legume *Lotus japonicus*: root explants as a source of large numbers of cells susceptible to *Agrobacterium*-mediated transformation. *Plant Cell Rep.*, **21**, 771–777 (2003).
5. Kawaguchi, M.: *Lotus Japonicus* ‘Miyakojima’ MG-20: an early-flowering accession suitable for indoor handling. *J. Plant Res.*, **113**, 507–509 (2000).
6. Dilworth, R. J., Chua, K. Y., and Thomas, W. R.: Sequence analysis of cDNA coding for a major house dust mite allergen, Der f 1. *Clin. Exp. Allergy*, **21**, 25–32 (1991).
7. Thomas, W. R. and Smith, W.: House dust mite allergens. *Allergy*, **53**, 821–832 (1998).
8. Robinson, C., Kalsheker, N. A., Srinivasan, N., King, C. M., Garrod, D. R., Thompson, P. J., and Stewart, G. A.: On the potential significance of the enzymatic activity of mite allergens to immunogenicity. Clues to structure and function revealed by molecular characterization. *Clin. Exp. Allergy*, **27**, 10–21 (1997).
9. Takahashi, K., Takai, T., Yasuhara, T., Yuuki, T., Ohtake, Y., Yokota, T., and Okumura, Y.: Production of enzymatically and immunologically active Der f 1 in *Escherichia coli*. *Int. Arch. Allergy Immunol.*, **122**, 108–114 (2000).
10. Jen, G. C. and Chilton, M. D.: Activity of T-DNA borders in plant cell transformation by mini-T plasmids. *J. Bacteriol.*, **166**, 491–499 (1986).
11. Ohta, S., Mita, S., Hattori, T., and Nakamura, K.: Construction and expression in Tobacco of a β -glucuronidase (GUS) reporter gene containing an intron within the coding sequence. *Plant Cell Physiol.*, **31**, 805–813 (1990).
12. Gamborg, O. L.: The effects of amino acids and ammonium on the growth of plant cells in suspension culture. *Plant Physiol.*, **45**, 372–375 (1970).
13. Handberg, K., Stiller, J., Thykjaer, T., and Stougaard, J.: Transgenic plants: *Agrobacterium*-mediated transformation of diploid legume *Lotus japonicus*, p. 119–128. In Celis, J. E. (ed.), *Cell biology: a laboratory handbook*. Academic Press, New York (1994).
14. Murray, M. and Thompson, W. F.: Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.*, **8**, 4321–4325 (1980).
15. Southern, E. M.: Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.*, **98**, 503–517 (1975).
16. Frohman, M. A., Dush, M. K., and Martin, G. R.: Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA*, **85**, 3998–4002 (1988).
17. Hsu, C. H., Lin, S. S., Liu, F. L., Su, W. C., and Yeh, S. D.: Oral administration of a mite allergen expressed by zucchini yellow mosaic virus in cucurbit species downregulates allergen-induced airway inflammation and IgE synthesis. *J. Allergy Clin. Immunol.*, **113**, 1079–1085 (2004).
18. Chen, Z. L., Schuler, M. A., and Beachy, R. N.: Functional analysis of regulatory elements in plant embryo-specific gene. *Proc. Natl. Acad. Sci. USA*, **83**, 8560–8564 (1986).