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Evaluation for Efficient Hairy Root Production Assessed by GFP-gene Expression in Tomato Cultivars Inoculated with *Agrobacterium rhizogenes* MAFF07-20001

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Abstract

When leaf segments of a tomato cultivar 'Ponderosa' were inoculated with *Agrobacterium rhizogenes* MAFF07-20001 carrying the binary vectors pRi and pBI121/sGFP, adventitious roots were developed from calli formed at the edges of the segments. Primordial roots were obtained with green fluorescence under blue light and elongated vigorously on hormone-free medium without loss of the green fluorescence. They were easily distinguishable from the non-fluorescing roots on the same segments. Successful integration of the sGFP and *rol* C genes into the chromosome of tomato roots was confirmed by polymerase chain reaction and Southern hybridization. The present method enables us to evaluate the hairy root formation without subculture, isolation and DNA analysis. All commercial cultivars available in Japan (24 cultivars) and 14 breeding lines of tomato were tested by this method. All but two breeding lines produced the hairy roots. Thus, the present method is useful for hairy root production in tomato.

Key words: *Agrobacterium rhizogenes*, GFP, hairy roots, tomato cultivars.

Abbreviations

GFP, green fluorescent protein; PCR, polymerase chain reaction.

An *Agrobacterium*-mediated gene delivery is a commonly available technique for genetically transforming higher plants. On account of easy selection of hairy roots, we have used *Agrobacterium rhizogenes* carrying Ri-plasmid for transformation of melon (Toyoda *et al.*, 1991; Matsuda *et al.*, 1997), tobacco (Matsuda *et al.*, 2001a) and strawberry (Toyoda *et al.*, 1993). In melon, Matsuda *et al.* (2000) selected hairy root clones that produced fruity, aromatic flavor at higher level than in fresh melon fruits, and Sawabe *et al.* (2002, 2003) pointed out the possible use of these hairy roots as biological material to produce useful compounds. This method has been also applied to tomato cultivars to obtain useful hairy root clones, but it is not always effective in distinction from non-transformed adventitious roots frequently produced in non-infected, detached tomato leaves. Moreover, the efficiency of hairy root formation varied among the cultivars used. To solve these problems, it is of practical importance to clarify the response of major tomato (*Lycopersicon esculentum* Mill) cultivars against the strains of *A. rhizogenes* for establishing a method for effectively selecting true hairy roots. From this point of view, we attempted to use the *A. rhizogenes* strain containing binary vectors (Ri-plasmid and pBI121/sGFP) for inoculation of commercial tomato cultivars. The present paper describes the successful formation of GFP-expressing hairy roots, which enable us to easily select fluorescing hairy roots and to evaluate the efficiency of transformation of tomato cultivars by the strains of *A. rhizogenes*.

The strain MAFF07-20001 of *A. rhizogenes* (Toyoda *et al.*, 1991) was used in the present study MAFF07-20001 was transformed with the plasmid vector pBI121/sGFP, which was constructed by

replacing the GUS gene of the plasmid vector pBI121 (Clontech, CA, USA) with the synthetic green fluorescent protein gene (sGFP(S65T)) (Chiu *et al.*, 1996). The sGFP gene was provided by Professor Dr. Y. Niwa, University of Shizuoka, Shizuoka, Japan. The integration of the plasmid into bacterial cells was conducted by electroporation according to the method previously described (Tanaka *et al.*, 1993). Transformed bacteria were screened in the presence of 25 mg l^{-1} of kanamycin, pre-cultured overnight and suspended in 10 mM $MgSO_4$ to give final density of 10^8 cells ml^{-1} as an inoculum.

Commercial cultivars and our breeding lines of tomato used in the present study are listed in **Table 1**. Seeds were germinated on a filter paper in a moistened petri dish, then hydroponically cultured for one month by the method reported previously (Nonomura *et al.*, 2001). The leaflets of the fifth-leaves were detached from the one-month-old seedlings and inoculated with *A. rhizogenes*. Inoculation was carried out according to the previous method (Toyoda *et al.*, 1991) with a slight modification. Namely, detached leaves were rinsed with sterile water, cut horizontally into two segments and dipped in an inoculum for 5 to 10 min. Inoculated leaves were stuck vertically into agar-solidified, sucrose-free MS medium (Murashige and Skoog, 1962) and incubated at 26℃ under continuous illumination of fluorescent lamp (3,000 lux). Induction of calli and hairy roots in inoculated leaf segments were recorded on a daily base for three weeks. GFP fluorescence in adventitious roots was observed under an Olympus fluorescence microscope BX-60 (IB excitation with BP460-490 excitation filter and BA510IF absorption filter).

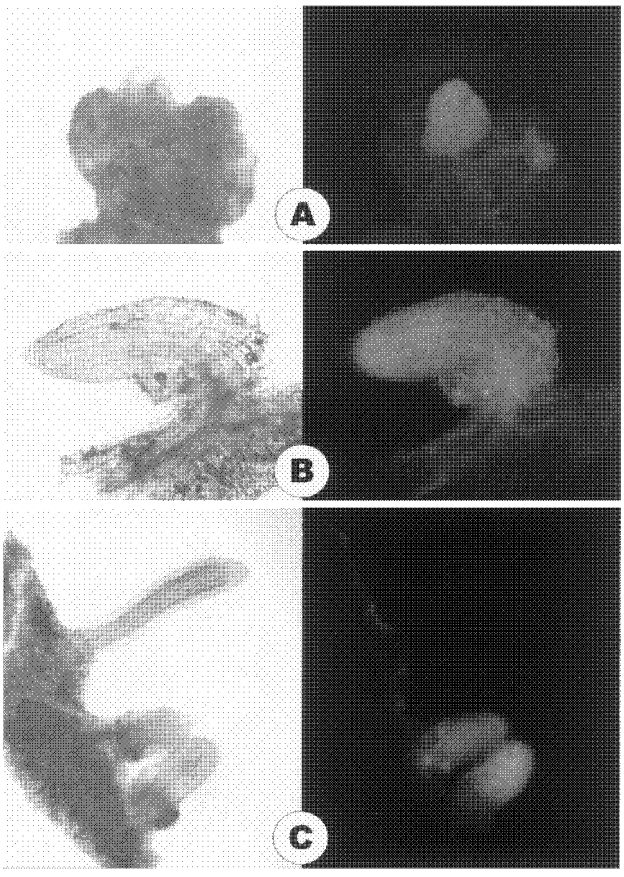

Fig. 1 Light (left) and fluorescent micrographs (right) of GFP-expressing hairy roots from calli produced by tomato leaf segments inoculated with *A. rhizogenes* MAFF07-20001 carrying binary vectors pRi and pBI121/sGFP. Photographs were taken at the same sites of inoculated leaves (cv. 'Ponderosa') under light and fluorescent fields of microscope. (A) and (B), GFP-fluorescing roots developed from abundantly proliferated calli (10 days after inoculation) and slightly proliferated calli (13 days after inoculation), respectively; (C), GFP-fluorescing hairy roots and non-fluorescing adventitious root developed from different calli induced in the same leaf segment (18 days after inoculation).

Prior inoculation of all tested tomato cultivars, we attempted to observe morphological changes in inoculated leaf segments of 'Ponderosa' that used routinely as a standard cultivar in our laboratory. First, calli were induced at the edge of excised leaf segments 10 to 12 days, and then the differentiated primordial root tips with green fluorescence 13 to 15 days after inoculation (**Fig. 1A**). These fluorescing primordial roots vigorously elongated on hormone-free medium (**Fig. 1B**). As shown in **Fig. 1C**, the green-fluorescing roots were distinguishable from non-fluorescent adventitious roots under blue light irradiation. In addition, other tomato cultivars also produced hairy roots in the same manner shown in Ponderosa, although the efficiency of hairy root induction varied among them. Thus, indicating that callus induction and subsequent development of hairy roots in tomato cultivars are recognizable in response to the inoculation with the present isolate.

To confirm successful integration of both sGFP and *rol* C genes into the chromosome of fluorescing roots, chromosomal DNA was extracted from bacteria-free fluorescing roots by CTAB method (Rogers *et al.*, 1985) and used for PCR. The fluorescing roots were subcultured for three months at a 10-day interval and used for the experiment. Non-fluorescing roots (3 to 4 cm long) were detached from leaf segments, surface-sterilized and homogenized for DNA extraction. PCR was conducted according to the previous method (Matsuda *et al.*, 2001a), with two sets of PCR primers (PGs/PGa, 5'-ATGGTGAGCAAGGGCGAGGAG-3'/5'-TTA-

Table 1 Evaluation of tomato cultivars for production of GFP-expressing hairy roots in leaf segments inoculated with *Agrobacterium rhizogenes* [1)]

Cultivars	Producers[2)]	Number of leaf segments used for inoculation (A)	Total number of roots with		Averaged number of GFP-fluorescing roots per segment (B/A)
			no fluorescence	green fluorescence (B)	
Kiomaru	MA	30	1	10	0.33
Ojyu	TA	160	39	35	0.22
K-29	KU	108	21	23	0.21
Toyomasa	SA	30	3	6	0.20
Putit	TA	225	63	44	0.20
Chibikko	MA	148	16	26	0.18
House Momotaro	TA	452	137	83	0.18
Fukuju Ni-gou	TA	151	14	25	0.17
Pepe	TA	141	26	24	0.17
Ponderosa	TA	274	45	45	0.16
Chika	TA	170	49	28	0.16
Corona	MA	233	18	36	0.15
K-30	KU	241	21	33	0.14
Odoriko	SA	288	34	40	0.14
K-54	KU	93	20	12	0.13
Kyouryokuminori	MA	227	73	30	0.13
Yellow Pico	TA	143	6	17	0.12
Twinkle Sweet	MA	137	10	17	0.12
K-28	KU	94	7	11	0.12
K-37	KU	114	13	14	0.12
Kyouryoku Beiju Ni-gou	TA	169	22	21	0.12
K-50	KU	100	17	12	0.12
Kyouryoku Beiju	TA	212	42	25	0.12
Momotaro	TA	291	13	32	0.11
K-31	KU	80	4	9	0.11
Hikari Fukuju	MA	70	4	7	0.10
Saturn	TA	127	9	13	0.10
Hakko	MA	84	6	8	0.10
Momotaro Eight	TA	174	19	18	0.10
Minicarol	AT	96	12	10	0.10
Home Momotaro	TA	205	26	20	0.10
Pico	TA	60	8	6	0.10
Yubi	MA	60	11	6	0.10
Moneymaker	TE	85	2	8	0.09
Coco	TA	192	21	18	0.09
Momotaro York	TA	180	24	16	0.09
K-98	KU	66	14	6	0.09
Momotaro T-93	TA	133	5	10	0.08
Zuiei	SA	62	3	5	0.08
Hokin Fukuju	TO	223	21	17	0.08
Marryroad	SA	135	14	11	0.08
Sekaiichi	MA	113	8	8	0.07
Shubi	MA	30	2	2	0.07
K-97	KU	86	7	6	0.07
K-86	KU	126	16	9	0.07
Toyofuku	SA	130	12	8	0.06
Firstpower	SA	66	3	3	0.05
Yellow Pear	TA	195	16	9	0.05
Sugarlamp	AT	40	16	2	0.05
Puti Eru	TO	46	1	2	0.04
K-84	KU	146	6	6	0.04
Red Pear	TA	155	11	6	0.04
LS-89	SA	79	0	1	0.01
K-83	KU	178	3	2	0.01
K-91	KU	160	0	0	0.00
K-92	KU	173	4	0	0.00

[1)] The strain MAFF07-20001 of *A. rhizogenes* containing binary vectors pRi and pBI121/sGFP was used for inoculation of excised leaf segments of tomato cultivars. Root induction was recorded on daily basis during the entire period of incubation (for 3 weeks).

[2)] TA, Takii Seeds, Kyoto, Japan; MA, Marutane Seeds, Kyoto, Japan; SA, Sakata Seeds, Yokohama, Japan; TO, Tohoku Seeds, Utsunomiya, Japan; AT, Atariya Noen, Chiba, Japan; TE, Thomas Etty, Esq., United Kingdom; KU, Kinki University, Nara, Japan. K-28 to K-98, breeding lines of tomato in Kinki University.

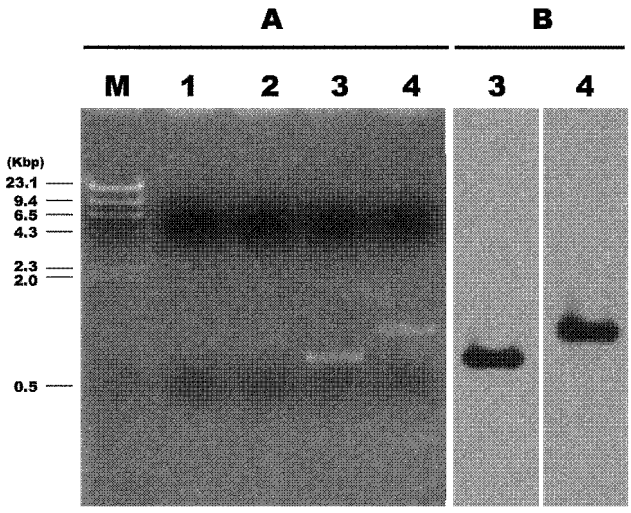

Fig. 2 Agarose-gel electrophoresis (A) and Southern hybridization (B) of PCR-products amplified from chromosomal DNA of non-fluorescing (lanes 1 and 2) and GFP-fluorescing roots (lanes 3 and 4). The PCR products were hybridized with the sGFP gene (lane 3 in B) or *rol* C gene (lane 4) as a probe.

CTTGTACAGCTCGTCCATGCC-3'; PRs/PRa, 5'-GAAGGAGTCGTGGCTAGTTAAGTGC-3'/ 5'-AGCTACTGCCATCACTCCATTCCAAA-3') specifically amplifying the sGFP and *rol* C genes, respectively. The primers were constructed on the basis of their nucleotide sequences reported. PCR products were electrophoresed on 0.9% agarose gels and transferred to nylon membrane for Southern hybridization. The sGFP and *rol* C genes clipped out of the original plasmids were labeled and used as probes for hybridization. Designated DNA products were amplified from chromosomal DNA of fluorescing roots and positively hybridized with the original genes used as probe (**Fig. 2**). In this experiment, ten fluorescing and non-fluorescing roots were selected randomly for this assay. The result confirmed that all of fluorescing roots tested were successfully integrated with both genes, however, neither DNA amplification nor hybridization signal was detected in all non-fluorescing roots. The non-fluorescing roots that integrated only the *rol* C gene into the chromosome were not detected in this study. Thus, the present study indicates that green-fluorescing roots were produced as the result of simultaneous integration of the sGFP and *rol* C genes on the binary vectors into the host plant chromosome, and the integrated genes were stably propagated during the subculture of hairy roots.

The present method enables us to effectively examine tomato cultivars for their production of hairy roots under blue light irradiation, without conducting the isolation, subsequent culture and DNA analysis for adventitious roots formed on inoculated leaf segments. **Table 1** summarized the hairy root production by commercial tomato cultivars and our breeding lines infected with *A. rhizogenes* containing the binary vectors. Although a few breeding lines ('K-91' and 'K-92') were resistant to the infection by MAFF07-20001, all other tomato cultivars showed susceptibility to the strain with some variation. Such a variation was often detected in susceptible tomato cultivars, when they were inoculated with bacterial (Nonomura *et al.*, 2001) and fungal pathogens (Matsuda *et al.*, 2001b). The different susceptibility of the cultivars to the pathogens was considered to be due to different genetic background of host plants (Agrios, 1988; Erickson *et al.*, 1990) or pathogenicity level of the pathogens (Anderson and Moore, 1979). Actually, the different strains (MAFF07-20002, MAFF03-01724, ArM-123, ArM-248) of *A. rhizogenes* failed to cause the hairy root production in these tomato cultivars (data not shown). In the present study, almost all tomato cultivars available in Japan were included, and eventually they could be effectively inoculated with the strain of *A. rhizogenes* to produce hairy roots. Convincingly, the present study provides basic information for transformation of tomato cultivars by the *A. rhizogenes*-mediated gene delivery.

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