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Effective Combination of *Agrobacterium tumefaciens* Strains and Ti Plasmids for the Construction of Plant Vector System

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The purpose of this study is to obtain the most efficient combination of *Agrobacterium tumefaciens* strains and Ti plasmids for the construction of dicotyledonous plant vector system. Ti plasmid-curing *A. tumefaciens* A136 and KU12C3 were transformed with four kinds of Ti plasmids, pTiBo542, pTiA6, pTiKU12 and pTiAch5, respectively. The stems of 28 species of dicotyledonous plants were then inoculated with these transformants and examined for crown gall formation. The different combination of *A. tumefaciens* strains and Ti plasmids showed quite a difference in terms of the crown gall formation. *Agrobacterium* strains A136 and KU12C3 have a same plant host range in case that both strains harbour the same kind of Ti plasmid, pTiBo542 or pTiAch5. However, the above-mentioned both strains have quite different host range in the event of containing the same Ti plasmid, pTiKU12 or pTiA6. In case that KU12C3 contains pTiA6 or pTiKU12, this strain has a wider plant host range than A136. The plant host range of pTiBo542 is the widest, followed by pTiA6, pTiKU12 and pTiAch5. Twelve plants among 28 tested plants are not transformed by any virulent *Agrobacterium* strains used in this study. In conclusion, *A. tumefaciens* KU12C3 and A136 harboring pTiBo542 showed the widest host range for transforming dicotyledonous plants. Also, it was acertained that the host range of Ti plasmids is affected by chromosomal level.

Keywords : plant transformation, *A. tumefaciens*, host range

The gram negative soil bacteria *A. tumefaciens* cause the plant disease crown gall (Lippincott *et al.*, 1981; De Cleene, 1985; Bytebier *et al.*, 1987; Raineri *et al.*, 1990). Crown gall is induced by tumor inducing (Ti) plasmid (Zeanen *et al.*, 1974) which embrace about 200 kb of DNA. T-DNA (transferred DNA) within Ti plasmid (Albright *et al.*, 1987) and *vir* region is essencial for crown gall formation. T-DNA which is surrounded by 25 bp imperfect direct repeats (Barker *et al.*, 1983: Simpson *et al.*, 1982) becomes integrated into the plant cell nuclear DNA. Besides the T-DNA, the Ti plasmid contains *vir* (virulence) region which has a size of about 30-40 kb and involved in T-DNA processing and transfer (Zambryski *et al.*, 1989). The *vir*-region of octopine Ti plasmid is consisted of eight operons, *vir*A to *vir*H. While mutations in the *vir*A, *vir*B, *vir*D, and *vir*G operons eliminate tumor formation on all plants species, mutations in the other loci (*vir*C, *vir*E, *vir*F, and *vir*H) lead to a restrictions in plant host range or to an attenuation of tumorigenecity (Hooykaas *et al.*, 1992, 1994; Lundquist *et al.*, 1984; Stachel and Nester, 1986). VirA and VirG together mediate the induction of the other *vir* operons in the presence of plant phenolic compound such as acetosyrigone (Stachel *et al.*, 1985; Winans *et al.*, 1992). Both the *vir* region and T-DNA contain genes that influence the plant host range of tumorigenesis. The strains which contain a wide host range (WHR) Ti plasmid cause tumors on a large variety of plants, while the limited host range (LHR) strain, isolated from *Vitis*, is restricted to a few plant species (Panagopoulos and Psallidas, 1973). Chromosomally located loci involved in attachment of *Agrobacterium* to the plant cell include *chv*A, *chv*B and *psc*A (*exo*C) (Stachel *et al.*, 1985).

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Table 1. Strains used in this study

Agrobacterium Strains	Genotype or Plasmids	Source
A136	Ti plasmid cured strain of strain C58	Watson(1975) *J. Bacteriol.* 123: 255-264
KU12C3	Ti plasmid cured strain of strain KU12	Ha(1993) Kor. *J. Microbiol.* 32(1): 53-59.
KU911	A136 containing pTiKU12	Lee(1993) PhD thesis
KU912	A136 containing pTiAch5	Ha(1993) Master thesis
A348	A136 containing pTiA6	Garfinkel(1980) *J. Bacteriol.* 144: 732-743.
A281	A136 containing pTiBo542	Komari(1986) *J. Bacteriol.* 166: 88-94
KU323	KU12C3 containing pTiKU12	in this study
KU324	KU12C3 containing pTiAch5	in this study
KU325	KU12C3 containing pTiA6	in this study
KU326	KU12C3 containing pTiBo542	in this study

Mutants in these loci are avirulent on many plant species (Douglas *et al.*, 1982; Douglas *et al.*, 1985). Therefore, host range determinants may be present not only in Ti plasmid but also in chromosomal DNA of *Agrobacterium tumefaciens*.

In previous research, *Agrobacterium tumefaciens* KU12 harbouring pTiKU12 (Lee, 1993; Lee, 1995) showed wider host range than A348 containing pTiA6 (Jun *et al.*, 1990). We have constructed various recombinated virulent *Agrobacterium tumefaciens* strains using two aviruent *Agrobacterium tumefaciens* strains, A136 and KU12C3 and four different Ti plasmids, pTiKU12, pTiAch5, pTiA6 and pTiBo542. In order to develop wide host range plant vector system, we investigated the effects of the recombination of various *Agrobacterium tumefaciens* strains and Ti plasmids on the tumor formation activity.

MATERIALS AND METHODS

Strains, media and plasmids

Agrobacterium tumefaciens strains and Ti plasmids uesd in this study are listed in Table 1. Bacterial strains were grown on MG/L or AB medium (Chilton *et al.*, 1974). AB-N and/or AB-N+ opine medium (Ha *et al.*, 1994) were used for the selection of transformants, KU323, KU324, KU325 and KU326.

Agarose, ethidium bromide, lysozyme, carbenicilline, kanamycine and octopine used in this experiment were purchased from Sigma Chemical Co. (USA).

Growth of plants

Kalancöe diagremontiana was obtained from E. W. Nester (University of Washington, USA). *Nicotiana tabacum* was obtained from Korea Institute of Science and Technology. Other plants were purchased from Seoul Seed Co., Ltd. in Korea. Plants were grown in soil consisted of a 5 : 1 mixture of vermiculite and peat moss at 30±5°C.

Preparation of plasmid DNA

Small and/or large scale preparations of plasmid DNA from *Agrobacterium tumefaciens* were carried out by the method of Birnboim and Doly (1979) and of Marco *et al.* (1982) with a slight modification, respectively.

Direct transformation of Agrobacterium

The direct transformation of *Agrobacterium* was performed by the freeze-thaw method (An, 1987). *Agrobacterium* strain was grown overnight in 5 mL of YEP medium (Yeast Extract 10 g, Peptone 10 g, NaCl 5 g/1 L, pH 7.0) at 28°C with vigorous shaking. 2 mL of the overnight culture was innoculated in 50ml of YEP medium and incubated further until the cells are grown to A_{600} of 0.5. After incubation, the cells were chilled on ice and harvested by centrifugation at 5,000 rpm for 5 minutes at 4°C in Sorvall SS34 rotor. The pellet was resuspended in 1 mL of cold 20 mM $CaCl_2$ solution and divided into 200 μL aliquot. To this 200 μL of competent cells, 1 μg of DNA was added, mixed well, and stored on

ice for 30 minutes. The mixture was rapidly freezed in liquid nitrogen bath and thawed by incubating in a 37°C water bath for 5 minutes. After addition of 1 mL YEP medium, the cells were incubated at 28°C for 2-4 hours with gentle shaking and the transformed cells were isolated using selective medium containing opine as specific nitrogen source instead of normal nitrogen source.

Virulence and plant host range assay

28 species dicotyledonous plants used in this study were listed Table 2. They were grown in green house for 4~5 weeks at 25°C±3°C. Virulence was assayed on stems as previously described (Garfinkel and Nester, 1980). Plants were kept in shadow area for 3-4 days and then transferred normal condition. Tumor symptoms were showed 2 weeks after inoculation. Virulence was scored as positive or negative based on the presence or absence of tumorous symptoms 30 days after inoculation.

Table 2. Plant species used in host range experiments

Scientific name	Common name
Phaseolus vulgaris var. *humils*	Haricot
Pharbitis nil.	Morning glory
Lycopersicon esculentum	Tomato
Zinnia elegans	Zinnia
Nicotiana tabacum	Tabacco
Kalancoe diagremmontiana	Mother-of-thousands
Cucurbita moschata	Pumpkin
Tagetes erecta	French merigold
Calendula officinalis	Calendula
Fagopyrum esculentum	Buck wheat
Mirrabilis jalaps	Marvel-of-Peru
Helaianthus annus	Sunflower
Brassica juncea	Leaf mustard
Cucumis melo var. *makuwa*	Melon
Solanum tuberrosum	Potato
Cucumis sativus	Cucumber
Solanum melongena	Eggplant
Ipomoea batatas	Sweat potato
Beta vulgaris var. *cicla*	Red beet
Celosia argentea	Cockscomb
Gossypium indicum	Cotton
Salvia officinalis L.	Salvia
Gomphrena globosa	Globe amaranth
Petunia hybriide vilm	Petunia
Lagenaria leucontha var. *gourda*	Calabash
Artemis princeps var. *orientalis*	Mugwort
Capsicum annnuuum L.	Red pepper
Portulaca grandiflora	Ross moss

RESULTS AND DISCUSSION

Assay system for comparison of virulences of *Agrobacterium* strains

In a search for a rapid, simple way of testing the virulence levels of *Agrobacterium tumefaciences* strains, the stems of 28 dicotyledonous plants were infected with ten *A. tumefaciens* A136, KU12C3, KU911, KU912, A348, A281, KU323, KU324, KU 325, and KU326 (Table 3). *Agrobacterium* A136 and KU12C3 were used as control. Tumors incited by some strains appeared 1 to 2 weeks earlier than those incited by the other strains among 28 plants. The speed of tumor growth differed with plants or strains. As a result, after 35 days, the tumor incited by A348 on the stem of morning glory was larger than those incited by KU911 or KU323 on the same plant (Fig. 1), and the tumor incited by KU326 on the stem of tobacco was larger than that by the same strain on morning glory stem (Fig. 1).

Effect of Ti plasmid on host range

The most powerful vectors for introducing foreign DNA into plant cells are based on the natural transforming system of *Agrobacterium* (Yanofsky *et al.*, 1985). Plant host range is determined by the particular Ti plasmid (Martin *et al.*, 1985, 1986; Tomashow *et al.*, 1981). Hood *et al.* (1986) demonstrated that a non-T DNA portion of the Ti plasmid is involved in the supervirulence phenotype of A281. The possibility is supported by a report that pTiBo542, when used as a source of *vir* genes, gave a higher frequency of transformation in the binary vector system than did other Ti plasmids (An, 1985). Jin *et al.* (1987) reported that 2.5-kilobase DNA fragment which contains *vir*G locus, as well as the 3' end of the *vir*B operon is responsible for the supervirulence phenotype.

In order to identify the Ti plasmid which has the widest host range, we constructed 10 virulent *Agrobacterium* strains which are consisted of different Ti plasmid and the same chromosomal background. A 136 and KU12C3 containing pTiBo542 were named A281 and KU326, respectively. A281 and KU326 showed wider host range in comparison with those *Agrobacterium* strains containing the other Ti plasmid, pTiA6, pTiKU12, or pTiAch5, indicating that pTiBo542 has the widest host range among the four tested Ti plasmids (Table 3).

Table 3. Responses of various plants to *A. tumefaciens* strains constructed by our laboratory

+ ····· Tumor formation
− ····· No tumor formation
NT ····· Not tested

Host plant	Formation of tumors by strain:									
	A136	KU12C3	A281	KU326	A348	KU325	KU911	KU323	KU912	KU324
Phaseolus vulgaris var. *humilis*	-	-	+	+	+	+	+	+	+	+
Pharbitis nil.	-	-	+	+	+	+	+	+	+	+
Lycopersicon esculentum	-	-	+	+	+	+	+	+	+	+
Zinnia elegans	-	-	+	+	+	+	+	+	-	-
Nicotiana tabacum	-	-	+	+	+	+	-	+	-	-
Kalancoe diagremmontiana	-	-	+	+	+	+	-	+	-	-
Cucurbita moschata	-	-	+	+	+	+	-	+	-	-
Tagetes erecta	-	-	+	+	+	+	-	+	-	-
Calendula officinalis	-	-	+	+	+	+	-	-	-	-
Fagopyrum esculentum	-	-	+	+	-	+	-	+	-	-
Mirabilis jalaps	-	-	+	+	+	+	-	-	-	-
Helaianthus annus	-	-	+	+	+	+	-	-	-	-
Brassica juncea	-	-	+	+	-	+	-	-	-	-
Cucumis melo var. *makuwa*	-	-	+	+	-	-	-	+	-	-
Solanum tuberrosum	-	-	+	+	-	-	-	-	-	-
Cucumus sativus	-	-	-	-	-	-	-	+	-	-
Solanum melongena	-	-	-	-	-	-	-	-	-	-
Ipomoea batatas	-	-	NT	NT	-	-	-	-	-	-
Beta vulgaris var. *cicla*	-	-	-	-	-	-	-	-	-	-
Celosia argentea	-	-	-	-	-	-	-	-	-	-
Gossypium indicum	-	-	-	-	-	-	-	-	-	-
Salvia officinalis L.	-	-	-	-	-	-	-	-	-	-
Gomphrena globosa	-	-	-	-	-	-	-	-	-	-
Petunia hybriide vilm	-	-	-	-	-	-	-	-	-	-
Lagenaria leucantha var. *gourda*	-	-	-	-	-	-	-	-	-	-
Artemis princeps var. *orientalis*	-	-	-	-	-	-	-	-	-	-
Capsicum annuum L.	-	-	-	-	-	-	-	-	-	-
Portulaca grandiflora	-	-	-	-	-	-	-	-	-	-
Total tumor	0	0	15	15	11	13	4	11	3	3

Effect of Bacterial strains on host range

Plant host range is determined in part by host species, bacterial strains (Stomp *et al.*, 1990; Bergmann *et al.*, 1992) and *vir* regulatory system (Winans *et al.*, 1992). We have compaired the host ranges of *Agrobacterium* A136 and KU12C3. The same fifteen plants *P. vulgaris* var. *humilis, P. nil, L. esculentum, Z. elegans, N. tabatum, K. diagremmontiana, C. moschata, T. erecta, C. officinals, F. esculentum, M. jalaps, H. annus, B. juncea, C. melo* var. *makuwa and S. tuberrosum* were susceptible to infection by A281 and KU326, indicating that A136 has a same host range as that of KU12C3 in case that both strains harbour pTiBo542 (Table 3). The same result was obtained in case that A136 and KU12C3 harbour pTiAch5.

However, two plants, *F. esculentum* and *B. juncea* were susceptible to infection by KU325, but not by A348. Also, seven plants, *N. tabacum, K. diagremmontiana, C. moschata, T. erecta, F. esculentum, C. melo* var. *makuwa* and *C. sativus* were susceptible to infection by KU323, but not by KU 911. The above-mentioned results indicate the following facts. First, the determination of host range is in part caused by chromosomal level of *Agrobacterium*. Second, *Agrobacterium* KU12C3 has a wider host range than that of A136 in case of harbouring pTiKU12 or pTiA6, respectively (Table 3). Third, the combination of Ti plasmids and *Agrobacterium* strains affects the host range of the virulent *Agrobacterium tumefaciens*. The high efficiency of transformation and the wide host range of *A. tumefaciens* strains are important considerations in applying this system to the genetic engineering of higher plants. Since *Agrobacterium* strains KU326,

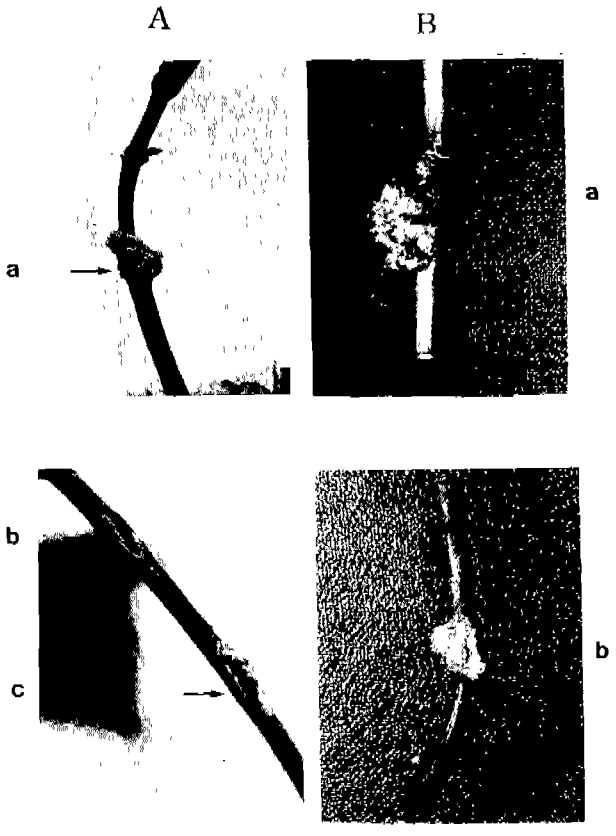

Fig. 1. Crown gall tumors incited by A348(A-a), KU 911(A-b) or KU323(A-c) on stems of *Pharbitis nil*, and by KU326 on stems of *Nicotiana tabacum*(B-a) and *Pharbitis nil*(B-b). Pictures were taken 35 days after inoculation.

KU325 and KU323 as well as A281 have a higher efficiency of transformation and a broader host range than the other stains, these strains may prove useful in transforming certain plants that are unsusceptible to infection by the other strains.

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