1-aminocyclopropane-1-carboxylate deaminase-producing *Agrobacterium tumefaciens* has higher ability for gene transfer into plant cells.

Running title: ACC deaminase enhances *Agrobacterium*-mediated gene transfer.

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ABSTRACT

Agrobacterium-mediated gene transfer is widely used for plant molecular genetics, and efficient techniques are required. Recent studies show that ethylene inhibits the gene transfer. To suppress ethylene evolution, we introduced 1-aminocyclopropane-1-carboxylate (ACC) deaminase into *Agrobacterium tumefaciens*. The ACC deaminase-producing *A. tumefaciens* showed higher ability of gene transfer in plants.

Agrobacterium-mediated gene transfer is widely used for plant molecular genetics and its applications (14). In particular, efficient systems of genetic transformation are required for plant functional genomics and molecular breeding to improve traits (20, 21). Recent studies showed that the ethylene is one of the negative factors for *Agrobacterium*-mediated gene transfer (1, 3, 5). Therefore, if *Agrobacterium tumefaciens* has ability to decrease the ethylene level in the host plant, it will increase the frequency of gene transfer. To suppress ethylene evolution from plant cells during co-cultivation, we introduced the 1-aminmocycroplopane-1-carboxylate (ACC) deaminase gene from *Pseudomonas* sp. ACP (7, 18) into *A. tumefaciens*. The enzyme cleaves ACC (ethylene immediate precursor) to α-ketobutyrate and ammonia, and as a result, ethylene level is decreased (4, 12, 16).

The ACC deaminase gene was amplified and cloned into pBBR1MCS-5 (10), a broad host-range plasmid, to generate a *lacZ::acdS* translational fusion (Fig. 1A). The plasmid was introduced into *A. tumefaciens* C58 (17) or C58C1Rif^R (2) by electroporation (19). The binary vector, pIG121-Hm involved in T-DNA transfer (6), was also harbored in *A. tumefaciens* C58C1Rif^R (pBBR*acdS*, pIG121-Hm). The ACC deaminase activity was assayed in *A. tumefaciens* C58C1Rif^R (pBBR*acdS*, pIG121-Hm) according to the method of Honma et al. (7). The amount of α-ketobutyrate in the reaction buffer was estimated from a standard curve based on a dilution of 10-400μM (detected at 340nm). The controls of this experiment were C58C1Rif^R (pBBRMCS-5, pIG121-Hm) and without substrate 200 μM of ACC in the reaction buffer. The accumulation of α-ketobutyrate was observed only

in the lysate from *A. tumefaciens* C58C1Rif^{R} (pBBR*acdS*, pIG121-Hm) with the substrate (Fig. 1B). Therefore, we succeeded in conferring ACC deaminase activity to *A. tumefaciens*.

Surface-sterilized melon (*Cucumis melo* L. var. *cantaloupensis* cv. Vedrantais) seeds were sown on half-strength of Murashige and Skoog's medium (MS) (13) and incubated at 25 ºC at 16h light condition for 5 days. Cotyledons from the germinated seedlings were transversely sectioned by hand into five pieces, and among them three internal pieces were inoculated. The segments were soaked into *A. tumefaciens* cell suspension of 10^7 cells ml^{-1} for 20 min, and the segments were placed on co-cultivation medium [MS containing 1.0 mg l^{-1} 6-benzylamino-purine, 2% glucose, 0.4% Gelrite (Wako, Tokyo, Japan), pH 5.5] in a gas vial at 16h light condition. Thirty melon cotyledon segments were inoculated with *A. tumefaciens* C58C1Rif^{R} (pBBR*acdS*, pIG121-Hm) per experiment. The experiments were repeated three times. After 24h of incubation, ethylene evolution from melon cotyledon segments was measured by gas chromatography (GC) (Fig. 2A). Compared to the uninoculation (control), ethylene evolution was enhanced from melon segment inoculated with *A. tumefaciens* C58C1Rif^{R} (pIG121-Hm) and C58C1Rif^{R} (pBBRMCS-5, pIG121-Hm). The application of 1 µM of aminoethoxyvinylglycine (AVG), an ethylene biosynthesis inhibitor reduced ethylene evolution from the inoculated segments. The inoculation of *A. tumefaciens* C58C1Rif^{R} (pBBR*acdS*, pIG121-Hm) suppressed ethylene evolution from melon cotyledon segments, and the level of ethylene accumulation

rate was the same as in the control and AVG treatment. These results indicated that *A. tumefaciens* with ACC deaminase activity reduced ethylene evolution from plants (Fig. 2A).

Three days after inoculation, the gene transfer was estimated (Fig. 2B). The pIG121-Hm has a reporter gene (35S-*uidA* intron) in the T-DNA region. Because the *uidA* reporter gene possesses an intron sequence, it can only produce active protein in plant cells, thereby making it a marker for gene transfer (15). Gene transfer was determined using fluorometric GUS assay according to Jefferson et al. (8). Melon segments inoculated with C58C1Rif^{R} (pIG121-Hm) and C58C1Rif^{R} (pBBR1MCS-5, pIG121-Hm) showed higher GUS activity than control. The higher GUS activity indicated that the gene was transferred. The addition of AVG (1 μM) increased GUS activity twice than without AVG. The inoculation with *A. tumefaciens* C58C1Rif^{R} (pBBR*acdS*, pIG121-Hm) showed approximately six times higher GUS activity than C58C1Rif^{R} (pIG121-Hm) inoculation. Thus, ACC deaminase enhanced the ability of gene transfer in *A. tumefaciens* (Fig. 2B).

Seeds of *Arabidopsis thaliana* (Columbia) were sterilized and grown at 22ºC for 7 days at 16h light condition after 4 days vernalization. Intact *A. thaliana* plants were dipped into *A. tumefaciens* C58 or A136 suspension (10^{7} cell ml^{-1}). A136 lacks Ti plasmid and T-DNA region and was used as control. The inoculated seedlings were blotted on sterile filter paper to remove excess suspension, and co-cultivated for 7 days at 16h light condition on MS. After co-cultivation, to eliminate the bacteria, the plants were washed in sterilized

water, and then incubated on MS containing 375 mg l^{-1} Augmentin for 3 weeks. Four weeks after inoculation with C58, C58 (pBBR1MCS-5) and C58 (pBBR*acdS*), green tumor had formed on the stem (Fig. 3A). The size of tumor was almost same, among infection (Fig. 3A). There were no tumors observed on plants inoculated with A136 (Fig. 3A, B). This result indicated that the tumor formation was formed by stable transformation (22). To estimate the genetic transformation efficiency, the number of *A. thaliana* forming green tumor was counted and the percentage were calculated. The fifteen intact *A. thaliana* seedlings were used each experiments and there were three independent repetitions. The percentage of tumor formed plant was 8.1±2.3%, 10.6±4.1% and 27.2±2.4% respectively, those inoculated with *A. tumefaciens* C58, C58 (pBBR1MCS-5 and C58 (pBBR*acdS*) (Fig. 3B). The tumor incidence was higher in the inoculation of ACC deaminase-producing strain. This result indicated that ACC deaminase activity increased the ability of stable transformation of *A. tumefaciens* (Fig. 3B).

Genetic transformation is a key technology for plant molecular breeding. Among several techniques of genetic transformation, *Agrobacterium*-mediated gene transfer is most frequently used techniques. Although large efforts have been made to establish efficient protocols of genetic transformation for plants of interest, still recalcitrant species/genotypes for genetic transformation exists such as cotton (11) and soybean (9). We succeeded in producing the *Agrobacterium* strain that is improved for the ability of gene transfer by providing an ability to reduce the ethylene level of the plant during co-cultivation. The

knowledge obtained in this study will provide a clue to overcome such problems in plant molecular breeding, which is producing transgenic plants in recalcitrant species and genotypes.

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LEGENDS of FIGURES

Fig. 1. Construction of the ACC deaminase-producing *Agrobacterium tumefaciens*.

(A) Plasmid construction for expression of ACC deaminase in *A. tumefaciens*. An HindIII and XbaI flagment (1kb) containing ACC deaminase gene in *Pseudomonas* sp. ACP gene was ligated into the HindIII and XbaI site of the broad host-range plasmid pBBR1MCS-5, resulting in pBBR*acdS*. The expression of the ACC deaminase gene *acdS* was under the control of the *lac* promoter. MCS means multiple cloning sites. (B) Detection of ACC deaminase activity in *A. tumefaciens*. The α-ketobutirate accumulation of the reaction buffer was measured according to Honma et al. (7). The triangle and cycle indicates the lysates from *A. tumefaciens* C58C1Rif^R (pBBR1MCS-5, pIG121-Hm) and C58C1Rif^R (pBBR*acdS*, pIG121-Hm), respectively. Closed and open symbols show samples with or without ACC in the reaction buffer, respectively. Bars indicate standard deviation (n=3).

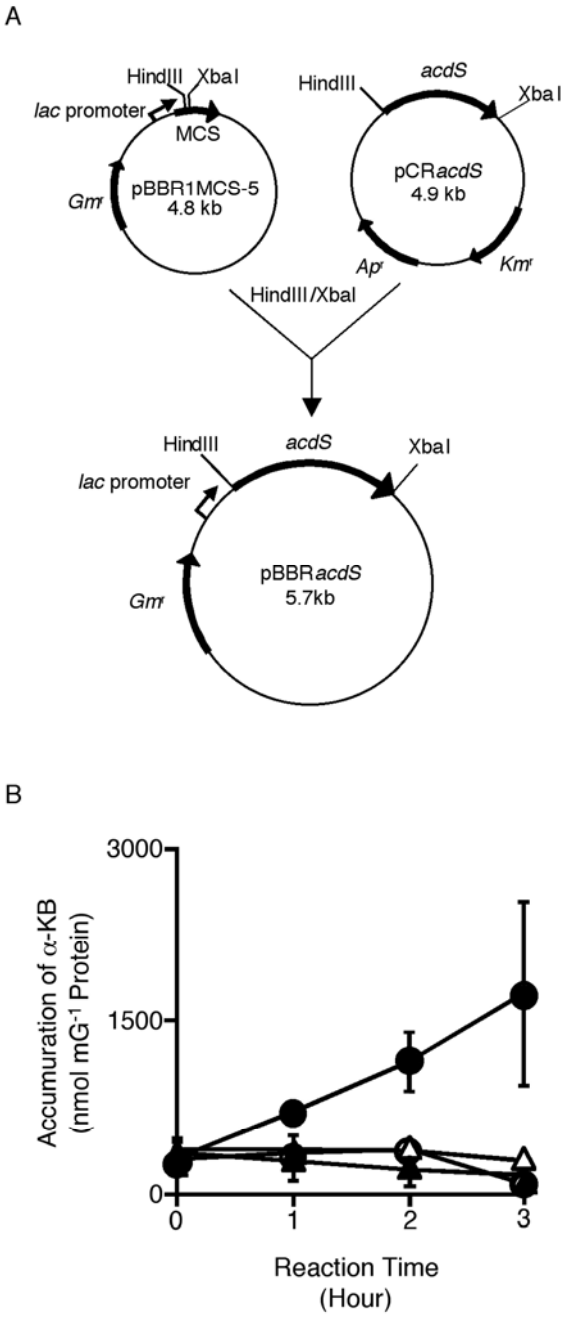
Fig. 2. Effect of ACC deaminase activity on ethylene evolution and gene transfer.

(A) Measurement of ethylene evolution. Accumulation of ethylene in the headspace was measured on a gas chromatograph. 121-Hm, 121-Hm/AVG, MCS-5 and ACDS indicate the inoculation with *A. tumefaciens* C58C1Rif^R (pIG121-Hm), addition of 1 μM AVG to the co-cultivation medium, C58C1Rif^R (pBBR1MCS-5, pIG121-Hm) and C58C1Rif^R (pBBR*acdS*, pIG121-Hm), respectively. Bars represent standard deviation (n = 3). The

characters show statistically significant differences (t-test; $p < 0.05$). (B) Quantification of gene transfer by GUS assay. Melon cotyledon segments were co-cultivated with three different *A. tumefaciens* strains for 3 days. 121-Hm, 121-Hm/AVG, MCS-5 and ACDS indicate the inoculation with *A. tumefaciens* C58C1Rif^R (pIG121-Hm), addition of 1 μM AVG to the co-cultivation medium, C58C1Rif^R (pBBR1MCS-5, pIG121-Hm) and C58C1Rif^R (pBBR*acdS*, pIG121-Hm), respectively. Bars represent standard deviation (n = 3). Different letters indicate statistically significant differences (t-test; $P < 0.05$).

Figure 3. The estimation of the genetic transformation-frequency by *acdS*-producing *A.tumefaciens.*

(A) Photographs of tumor formation on Arabidopsis stems. Pictures of tumors on stem of *A. thaliana* were taken four weeks after the inoculation. (B) The frequency of genetic transformation. C58, MCS5 and ACDS indicate the inoculation by *A. tumefaciens* strains, C58, C58 (pBBRMCS-5) and C58 (pBBR*acdS*), respectively. Bars indicate standard deviation (n=3). The characters represent statistically significant differences based on chi-square testing ($P <0.05$).


A
HindIII
XbaI
lac promoter
MCS
pBBR1MCS-5
4.8 kb
Gmr
HindIII
acdS
XbaI
pCRacdS
4.9 kb
Apr
Kmr
HindIII/XbaI
HindIII
acdS
XbaI
lac promoter
pBBRacdS
5.7kb
Gmr
B
3000
1500
0
Accumuration of α-KB
(nmol mG-1 Protein)
0
1
2
3
Reaction Time
(Hour)

1

2

3 Fig. 1

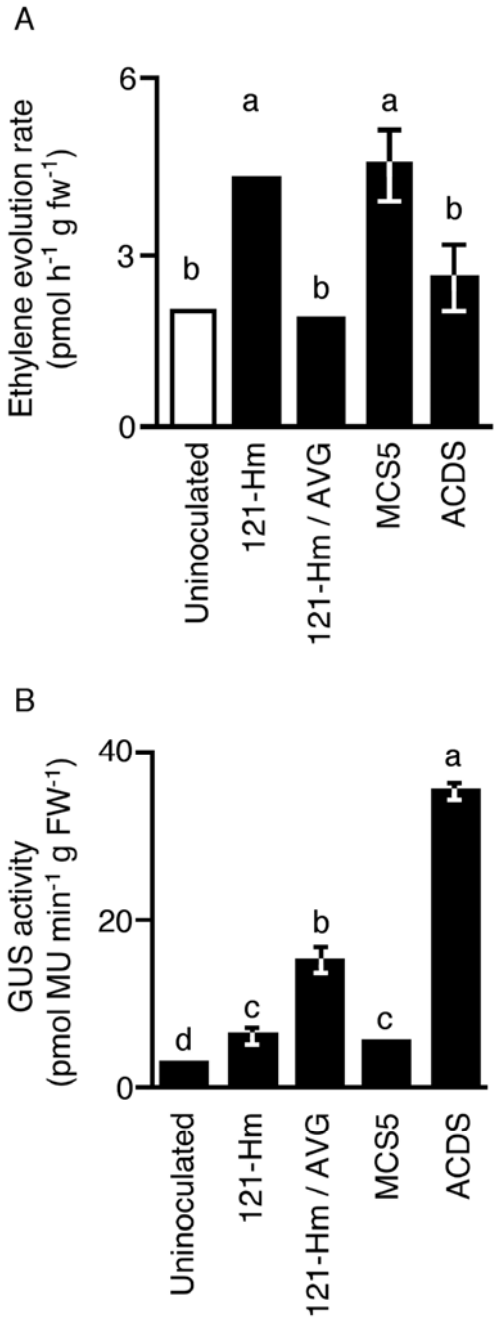

A
Ethylene evolution rate
(pmol h-1 g fw-1)
6
3
0
a
a
b
b
b
Uninoculated
121-Hm
121-Hm / AVG
MCS5
ACDS
B
GUS activity
(pmol MU min-1 g FW-1)
40
20
0
a
b
c
c
d
Uninoculated
121-Hm
121-Hm / AVG
MCS5
ACDS

Fig. 2

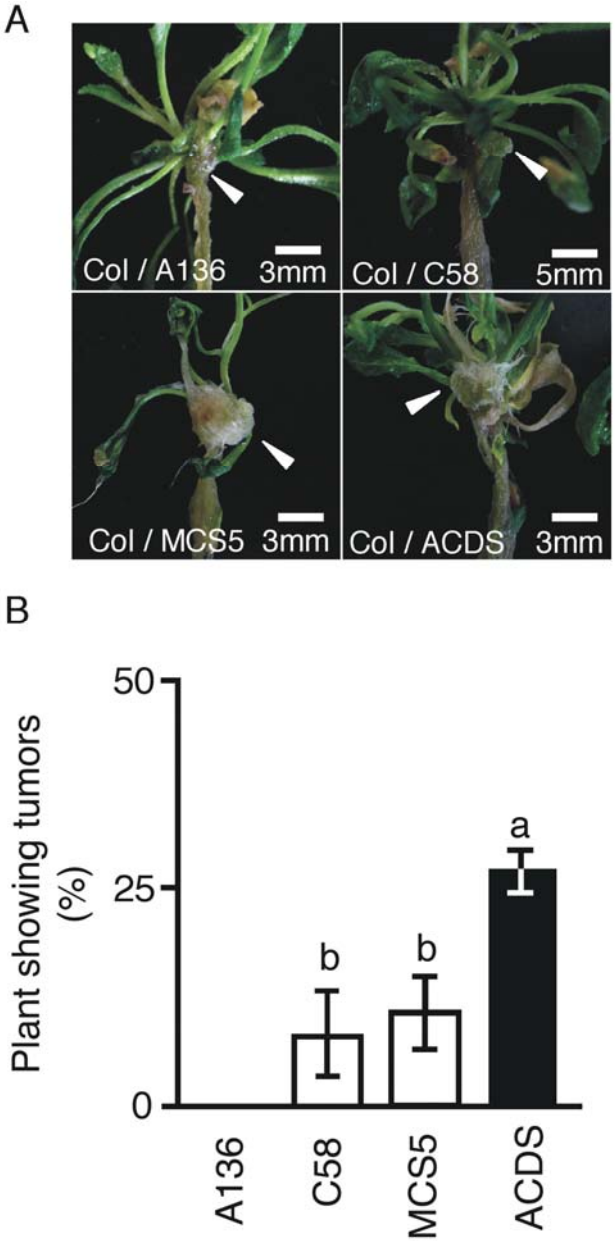

A
Col / A136
3mm
Col / C58
5mm
Col / MCS5
3mm
Col / ACDS
3mm
B
Plant showing tumors (%)
50
25
0
b
b
a
A136
C58
MCS5
ACDS

Fig. 3