The field testing report

Genetic characteristics investigation and biosafety assessment on transgenic orchid *Oncidesa* Gower Ramsey ‘Honey Snow MF-1’

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Field testing institution: Southern Biotechnology Center, Academia Sinica

Recipient plant: *Oncidesa* Gower Ramsey ‘Honey Angel’

Transgenic strain: *Oncidesa* Gower Ramsey ‘Honey Snow MF-1’

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Abstract

This report is an experimental result of genetic characteristics investigation and biosafety assessment on *Oncidesa* Gower Ramsey ‘Honey Snow MF-1’ (abbreviated as MF-1). Recently, RNA interference (RNAi) technology was employed to suppress carotenoid biosynthetic pathway gene ‘*phytoene synthase*’ of the recipient plant *Oncidesa* Gower Ramsey ‘Honey Angel’ and successfully modified floral color from yellow hue to white, to create the transgenic oncidium plant. MF-1 have been grown in biosafety greenhouse to investigate the genetic characteristics of its growth, flower morphology and genetic stability, and the results confirmed that their genetic and physiology morphological differences were similar to *Oncidesa* Gower Ramsey ‘Honey Angel’, and the expression level of the transgene was stable. Cross hybridization test by intra-species and inter-genus showed that no successful fertilizations were obtained. Moreover, the pollen sterility was demonstrated by the cytological evidences on microsporogenesis, i.e. chromosomal abnormally pairing, chromosomal asymmetric segregation, etc. Pollen germination test further confirmed MF-1 pollen lost its viability and were unable to germinate. Furthermore, assays on weediness ability, allelopathic effect, rhizospheric microbial antagonism, and *Agrobacterium* residue clearly revealed that MF-1 are potentially safe and without possible risk to environmental biodiversity. The assays on the gene flow from MF-1 to surrounding microbial, insects, and pathogens proved its safety. All the results indicated that the risk of *Oncidesa* Gower Ramsey ‘Honey Snow MF-1’ being a threat to the biodiversity is low.

Key words: oncidium orchid, genetic characteristics investigation and biosafety assessment, pollen sterility, RNA interference (RNAi) technology

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(1) Executive period and experimental location

A. The executive period

From April 2 until December 31, 2015.

From August 8 until September 12, 2017

B. Experimental location

The trial greenhouse is located at the Southern Biotechnology Center, Academia Sinica, Tainan, which is a government authorized facility with semi-confined equipment, and specific functional for the field testing. (http://www.as-bcst.sinica.edu.tw/wenshi/banfa.asp)

(2) Plant materials and growth condition

A. Recipient plant for gene transformation

(a) The recipient plant is *Oncidesa* Gower Ramsey 'Honey Angel'

(b) Taxonomic status: Plant Kingdom, Angiosperm Phylum, Monocotyledon Class, Asparagales Order, Orchidaceae Family, Oncidiinae, *Oncidesa* Gower Ramsey 'Honey Angel' (abbreviated as HA).

B. Transgenic oncidium orchid plant:

The transgenic orchid plant used in the GMO biosafety test is : *Oncidiesa* Gower Ramsey ' Honey Snow MF-1'(abbreviated as MF-1).

C. Growth Condition:

Both plants with vegetative stage (HA and MF-1), 50 pot-seedlings each, were selected for test at bolting stage and were grown in semi-confined greenhouse. The growth condition mimicked the natural condition, 25~30^{0}C/day, 20-25^{0}C/night; light intensity 250~350μmole/m^2.sec/14hr. Growth characteristics and flowering parameter were recorded.

Fig. 1 Plant morphology of *Oncidesa* Gower Ramsey 'Honey Angel' (HA, left) and transgenic *Oncidiesa* Gower Ramsey ' Honey Snow MF-1' (MF-1, right)

(3) Results of the genetic characteristics investigation

A. General growth characters and flower morphology of MF-1

The observation of growth rate, flowering time and flower morphology on *Oncidesa* Gower Ramsey 'Honey Angel' (HA, as control CK) and *Oncidesa* Gower Ramsey 'Honey Snow MF-1' (MF-1) in the semi-confined greenhouse was conducted from April 1 to December 31, 2014. There are similar physiological/morphological found between two varieties, except the flower pigment is different with yellow in HA, but white in MF-1 (Fig.1). The shelf life of both post-harvested flowers also showed no significant difference. This results indicated that the genetically modified point is restricted to petal tissues, and that no negative collateral effects occur on other tissues, which also suggests that the activity of *Pchrc* promoter is functioning in petal tissues.

B. The Pollination test and assays on pollen viability of MF-1

To confirm the pollen viability and the possibility of gene flow by distant pollination, we performed pollination test by hybridizing pollens of MF-1 with different orchid plants.

(a) Cross hybridization test by intra-species

In the intra-species with HA, data showed that no successful fertilizations were obtained (Table I).

Table I. Outcome of cross hybridization between HA and MF-1. Ten florets each were used to perform cross-pollination test.

	HA X MF-1 (donor)	**MF-1 X HA (donor)**
Hand pollination	10 florets	10 florets
Abortion	10 florets	10 florets

(b) Cross hybridization test by inter-genus with *Oncidium* plants

In inter-genus hybridization, HA and MF-1 hybridized with *Oncidium* Twinkle 'Red', *Oncidium* Kaizumic Delight 'Green Stone' (Fig. 2), it showed a negative data (Table II).

Fig. 2 Two oncidium orchid plants used for pollination test with MF-1 as the mode of inter-genus hybridization. Pollens of *Oncidesa* Gower Ramsey 'Honey Snow MF-1' were hybridized to *Oncidium* Twinkle 'Red' (left) and *Oncidium* Kaizumic Delight 'Green Stone' (right).

(c) Cross hybridization test by inter-genus

In addition, by using the outer-genus hybridization test with *Phalaenopsis Aphrodite* and *Phalaenopsis equestris*, it showed a negative data too (Table II).

Table II. Outcome of cross-hybridization by inter-species and inter-genus strategy among HA (as control CK), MF-1, *Oncidium* Twinkle 'Red', *Oncidium* Kaizumic Delight 'Green Stone' and *Phalaenopsis aphrodite* 'N2K01' (moth orchid)

	Oncidium Twinkle 'Red'	*Oncidium* Kaizumic Delight 'Green Stone'	*Phalaenopsis aphrodite* 'N2K01'
HA (doner)	2 florets used/2florets abortion	6 florets used/6 florets abortion	6 florets used/6 florets abortion
MF-1 (doner)	2 florets used/2florets abortion	6 florets used/6 florets abortion	6 florets used/6 florets abortion
Self-pollination	2 florets used/2florets abortion	2 florets used/2florets abortion	2 florets used/2 seed pods

(d) Investigation on meiosis, chromosomal behavior and microsporogenesis of MF-1

Cytological studies during microsporogenesis (process of pollen formation) was carried out to observe the meiotic abnormalities related to irregular chromosome in MF-1 under microscope (Fig 3 A-H). Therefore, a sample of the microspore mother cells undergoes meiosis to produce haploid microspores formation. In this investigation, the results of this experiment revealed that during the process of meiosis, microspore mother cells undergo gene transfer in which only part of chromosome were paired during this phase (Fig 3A), and homologous recombination were abnormally paired (Fig 3B, C). The process of abnormal chromosome segregation at the end of the meiosis is significantly disturbed, in which chromosome lag resulted in forming a chromosome bridge (Fig 3D-H). This phenomenon leads to the result of asymmetric segregation of chromosomes (Fig. 3, I- N). Therefore, after the end of meiosis, the number of chromosomes in each of the pollen cells produced by each sub-cell (microspores) might be different, and the genetic material composition was also incomplete, which may be one of the reason MF-1 is considered to be sterile.

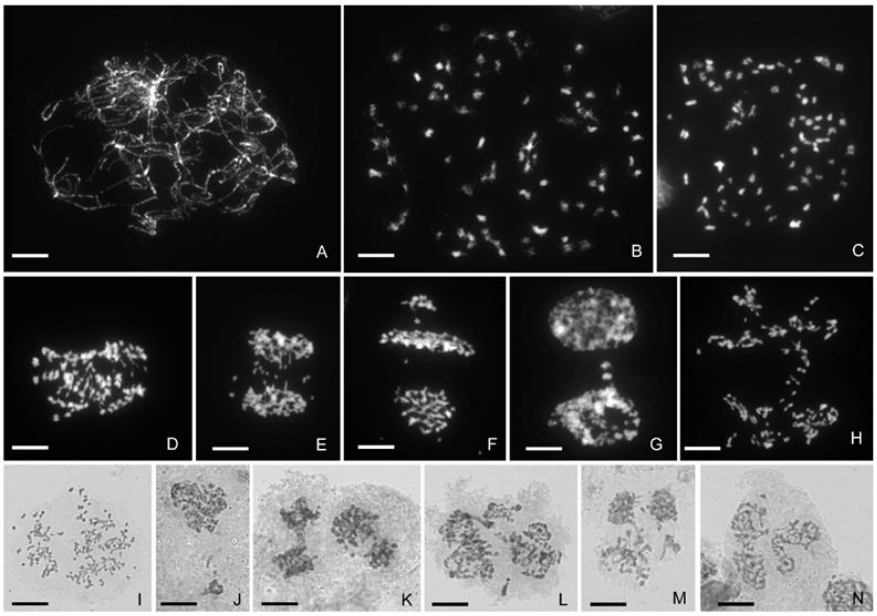


Fig. 3 The process of the microsporogenesis at meiosis in MF-1. In the pachytene stage, only part of the chromosomes are paired (A), homologous chromosomes are not normally paired (B, C), sub-chromosomal segregation is delayed to form a chromosome bridge (D-H), and uneven distribution of sub-chromosomal (I-N).

Normally, tetrad formation of four microspores is typical structure in the completeness of microsporogenesis. However, the abnormal end-products were generated (Fig. 4). Abnormal types of triad (Fig. 4D), tetrad (Fig. 4A-C,E), pentad-hexad (Fig. 4F-I) were formed. This suggest that the multiple ancestors of chromosomal origin caused chaotic pairing and unequal segregation. Consequently, microspores are not normally generated.

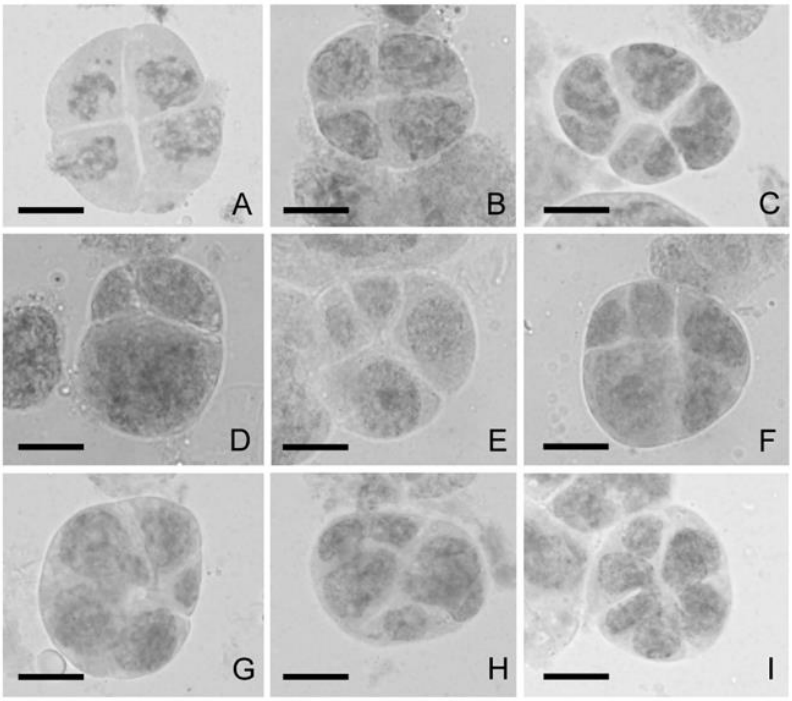


Fig. 4 Results of Chromosome segregation and cytoplasmic distribution (cytokinesis) during meiosis of MF-1 microspore mother cells. Variation of heterogeneous microspore size are as follows: the triad (D), the tetrad unequal number (A, B, C, E) and pentad-hexad (F-I).

Furthermore, the microscopic observations also showed that shapes of pollen grains are irregular and size varied(Fig.5 B), comparing to the uniform appearance in *Oncidium sphacelatum* (Fig.5 A, one of the ancestors). Overall, these cytological evidences will support the sterility of MF-1.

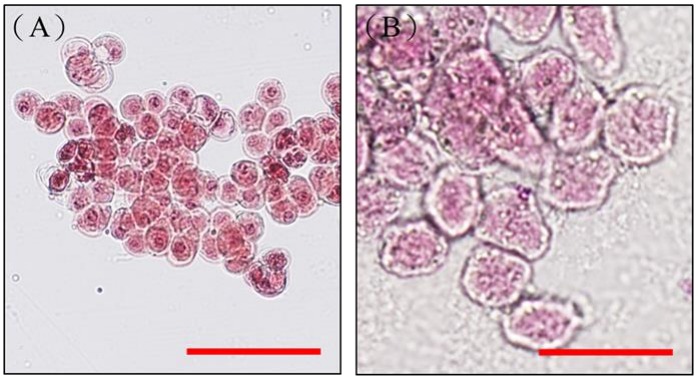


Fig. 5 Pollen cells of *Oncidium sphacelatum* (A, one of the ancestors as control) and MF-1 (B). Unequal cytoplasm and size occurring in MF-1. scale bar: 50μm, stained by acetocarmine solution.

(e) Pollen germination rate of MF-1

By using Brewbaker and Kwack medium (1963) supplemented with sucrose of 0, 1, 2, 3, 10% to test the ability of pollen germination. After different incubation period of 3, 10, 15 days, no germination was occurring in MF-1, the pollen of *Oncidium sphacelatum* (one of the ancestors) used as control, germinates in each kind of medium condition. Data demonstrated that pollens of MF-1 have lost viability completely, so that no pollen germination was found in the test (Table III). It clearly demonstrated that pollen cell of MF-1 lost viability and unable to germinate.

Table III. Pollen germination tested on BK medium supplemented with 0, 1, 2, 3, and 10% sucrose at 3, 10 and 15 days, 25°C. Pollen samples used were collected from MF-1 and *Oncidium sphacelatum*, which is one of the ancestors used as control.

		BK medium				
Sucrose (%)		0 %	1 %	2 %	3%	10 %
Oncsa. GR' Honey Snow MF-1	3 days	−	−	−	−	−
	10 days	−	−	−	−	−
	15 days	−	−	−	−	−
Sucrose (%)		0 %	1 %	2 %	3%	10 %
Onc. sphacelatum	3 days	−	+	+	++	+
	10 days	+	+	++	+++	+
	15 days	++	+++	+++	+++	+++

C. Gene expression levels and stability of MF-1

MF-1 was created by RNAi technology with the silence of *phytoene synthase* (*OgPSY*) RNA transcripts. This transgenic plant harbored T-DNA construct containing *hygromycin phosphotransferase* gene (*HPTII*, marker gene), and two DNA segments of *OgPSY*, which are inversely orientated forming hairpin mRNA. The hairpin mRNA is expressed only in petal tissue and not in roots and leaves. (Liu *et al.*, 2019). To confirm the stability of gene transformation and expression, The primer pair of *HPTII gene*: 5'-GATGTAGGAGGGCGTGGATA-3'/5'-CGTCTGCTGCTCCATACAAG-3'（size of PCR amplification is 621 bp）and the primer pair of *OgPSY*: 5'-AACTAGTTCTGGGCAATCTATGTGTGG-3'/5'-CACTAGTCCCATTAAATATATCCTCATCTG-3' were used for RT-PCR performance to detect the gene expression levels in various tissues of MF-1/HA(as control CK) plants during vegetative stage and flowering (reproductive) stage. As shown in Fig. 6, the RT-PCR data done with vegetative stage revealed *OgPSY* gene is constitutively expressed in root, pseudobulb and leaf of MF-1 and HA. However, *HPTII* gene is only shown in MF-1, while not in host plant. This indicated that T-DNA was specifically inserted in MF-1. On the other hand, *OgPSY* is not silenced in vegetative tissues of MF-1. Furthermore, Fig. 7 showed that *OgPSY* is specifically silenced in flowering tissues of MF-1, while not in host plant. Also, *HPTII* gene expression is only discovered in MF-1, while not in host plant. Taken together, the results identified that MF-1 used in the experimental test is a stable transgenic plant.

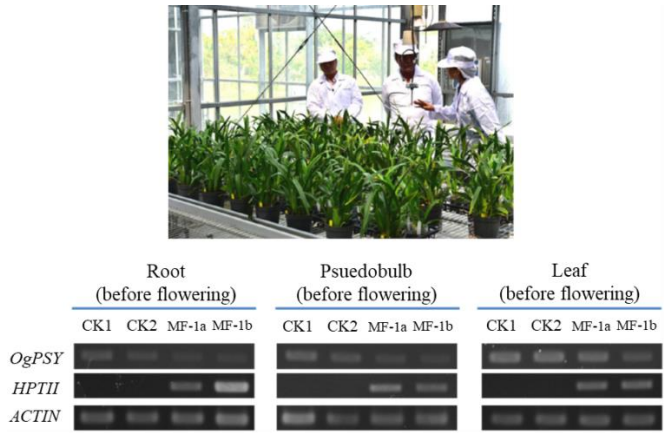


Fig. 6 Detection of gene expression level by RT-PCR in vegetative stage. The expression level of *OgPSY* and *HPTII* in root, pseudobulb and leaf tissue in vegetative stage were detected by RT-PCR. Vegetative plants grown in semi-confined greenhouse (upper panel). RT-PCR data (lower panel) revealed *OgPSY* expression constitutively in vegetative tissues of HA (CK1 and CK2) and MF-1 (MF-1a and MF-1b). *HPTII* is specifically expressed in MF-1. *ACTIN* used as internal control.

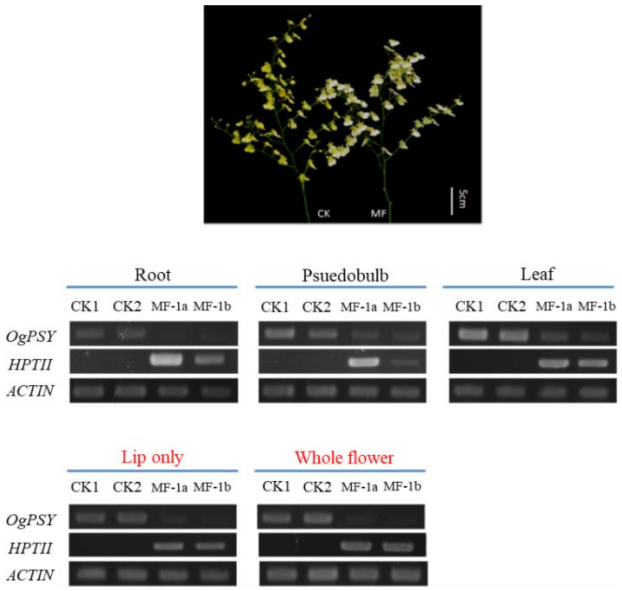


Fig. 7 Detection of gene expression level by RT-PCR in flowering stage. The expression level of *OgPSY* and *HPTII* in root, pseudobulb and leaf tissue in flowering stage (upper panel) were detected by RT-PCR. RT-PCR data (lower panel) revealed *OgPSY* expression constitutively in vegetative tissues of HA (CK1 and CK2) and MF-1 (MF-1a and MF-1b), but without in tissues of lip (shown by lip only and whole flower) of MF-1. *HPTII* is specifically expressed in MF-1. *ACTIN* used as internal control.

(4) Results of assays on biosafety assessment

A. The potential for weediness of MF-1

The risk of becoming a weed in the environment is one of the concerns on all transgenic plants. In this case of oncidium orchid, however, pollens of MF-1 are sterile. Moreover, the oncidium orchid is used to be cultivated in net-house and greenhouse by farmers. Therefore, there is little possibility of escapes from the confined cultivation space to outside places. Moreover, based on the past 30-year observation, thus far, no outside growth has been recorded. Additionally, as oncidium orchid plants are epiphytic with a sympodial growth habitat, there is no rhizome organ to propagate its seedling. Taken together, the weediness of MF-1 is very difficult.

B. The harmful effects on the targeting organisms by MF-1

Besides, because MF-1 does not contain (gene harboring) the insect- and pathogen-resistant genes for overexpression, it is not harmful to specific organisms. According to the regulation issued by COA on Aug. 22, 2007 (農糧字第 0961051654 號), transgenic plant without ectopic overexpression gene of insect- and pathogen-resistant genes is exempted from this test.

C. The harmful effects on the non-targeting organisms by MF-1

(a) Evaluation of the harmful effects on the non-targeting insects

MF-1 was tested in the semi-confined greenhouse during the 8-month field testing. There were not any visible visiting insect observed, but the common fruit fly. In the assay, the common fly was recognized as non-targeting insects (Fig. 8A). Several individuals were caught by glued paper and sampling to extract the genomic DNA. PCR assay was performed to monitored the gene flow horizontally. PCR data showed no detection of *HPTII* gene signal, indicating that no gene flow from MF-1 to fruit fly (Fig. 8B).

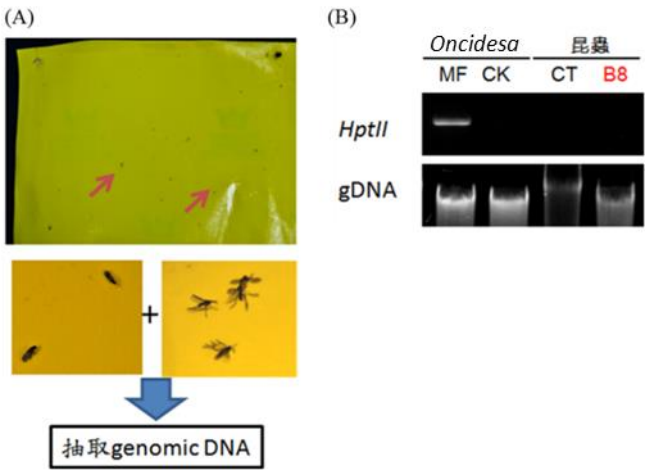
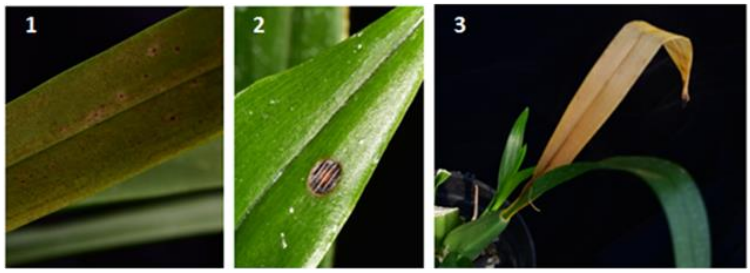


Fig. 8 PCR monitor for the *HPTII* gene flowing into genome of insects. (A) Collection of fruit fly sample in greenhouse, (B) PCR assay of *HPTII* gene in the genome of fruit fly, the gel electrophoresis shows no detection of *HPTII*. (CK: HA; MF: MF-1; B8: Fruit fly collected from MF-1 orchid; CT: Fruit fly collected from HA.)

(b) Evaluation of the harmful effects on non-targeting pathogen by MF-1

This test was performed in a semi-confined greenhouse under 30℃/20℃ day/night and without any pesticide application. There were not any visible pathogenic symptoms observed during the period. However, some symptoms, likely necrosis, occurred at the end of the test (Fig. 9A), and it showed that MF-1 is more resistant than HA (Fig. 9B). It seemed owing to MF-1 is younger in generation than HA. The infected diseases are owing to the reduced plant immunity in aged. It indicates that plants are usually resistant, when they are active in young stage.

(A)



(B)

Line	Disease 1		Disease 2		Disease 3	
	Infected plant No.(%)	Infection damage	Infected plant No.(%)	Infection damage	Infected plant No. (%)	Infection damage
CK	32/50 (64%)	15%	20/50 (40%)	21%	0/50 (0%)	0%
MF	0/50 (0%)	0	20/50 (40%)	16%	2/50 (4%)	20%

Fig. 9 Disease symptoms and susceptibility rates in MF-1 and HA. (A) Some symptoms, likely necrosis, occurred at the end of the test. (B) MF-1 was more resistant to Disease1 than HA, other Disease damage showed no significant difference. CK: HA; MF: MF-1.

(c) Evaluation of the harmful effects on rhizosphere microbial population by MF-1

A survey of the rhizosphere microbe community impacted by MF-1 was evaluated by using a plate culture method (Shiomi *et al.*, 1992). The results revealed that there are no significant differences in population of bacteria, and filamentous fungi between MF-1 and HA (Fig. 10). The results suggested that no toxic metabolite was produced from MF-1. In the root circle microbial assessment, if there is no significant difference in the number of colonies between genetically transferred and non-genetically transferred plants, it can be regarded as genetically safe as well as environmentally friendly plants. (Yu *et al.*, 2013a, 2013b; Oguchi *et al.*, 2014)

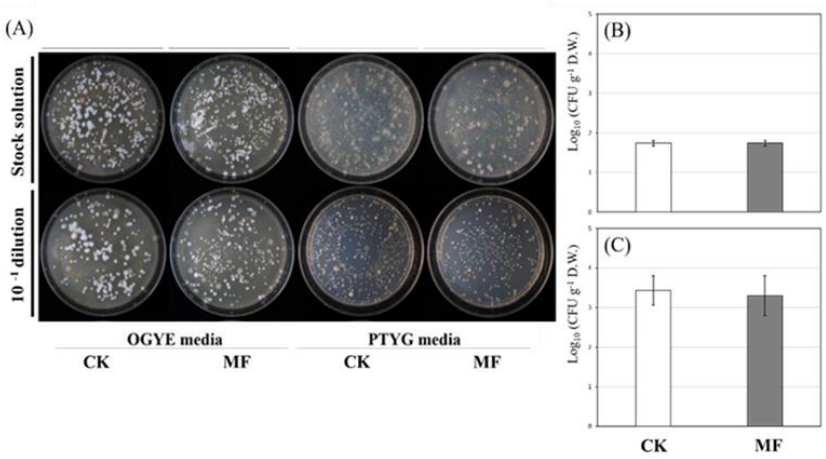


Fig. 10 A survey of rhizosphere microbial population. (A) Rhizosphere microbes, collected from MF-1 (MF) and HA (CK), were cultivated at 28^{o}C for three days by using the plate culture method on two media: OGYE and PTYG, respectively. OGYE (Oxytetracycline Glucose Yeast Extract) medium is mainly used to culture fungi, and the PTYG (Peptone Tryptone Yeast Extract Glucose) medium is used to culture bacteria. Colony-forming units of (B) fungi, and (C) bacteria were counted. The data were presented the average of three replicates ± SD from each tested. It did not show any significant difference between MF-1 and HA.

(d) Evaluation of the harmful effects on animals by MF-1

Basically there are few animals fed on oncidium orchid plants. Moreover, the cultivated oncidium orchids, such as *Oncidesa* Gower Ramsey and HA, are grown in net house and semi-confined facility by Taiwan farmers, and wild animals are prevented from the orchid farm. At most, on very rare occasion, only small snails or slugs can be observed in the poorly conditioned orchid farms (Fig. 11). Therefore, we collected snails and slugs that creeped on MF-1 and to check whether integrated T-DNA has been transferred from MF-1. Many studies have shown that it is hard to transfer plant genes to animals and microorganisms (horizontal gene transfer, HGT, Aeschbacher *et al.*, 2005; Sharma *et al.*, 2006; Keese, 2008; Xu *et al.*, 2009). This test can identify the possibility of gene flow from transgenic plants to visiting animals. The result confirms that the possibility of horizontal gene transfer hardly exists (Fig. 12, Table IV).

Fig. 11 Snails and slugs occasionally feed on the leaves of oncidium orchid.

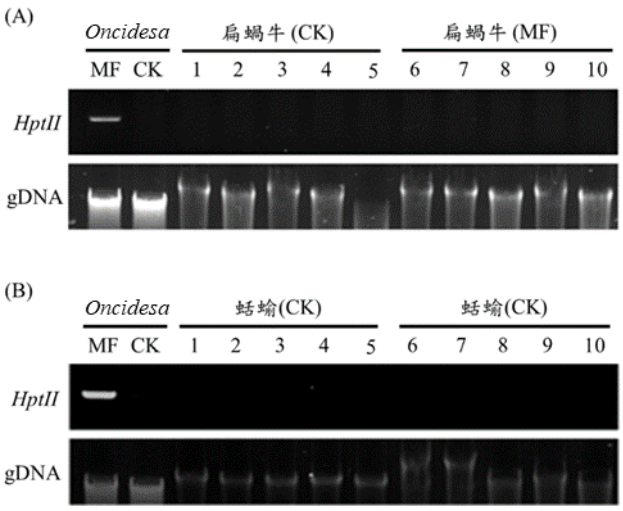


Fig. 12 Assay on the *HPTII* integration in the genomes of snails (A) and slugs (B). The results showed that all snails and slugs fed HA or MF-1 did not detect *HPTII* gene. (CK: HA; MF: MF-1)

Table IV. Harmful situation of feeding snails and slugs with oncidium orchids for three monthes.

Line	Bite plant No. (%)	Bite damage (%)
CK	5/5 (100%)	42%
MF-1	5/5 (100%)	44%

Total 30 snails and slugs were fed on living oncidium orchid plants of CK(HA) and MF-1 (n = 5 each). Data of damage were recorded at 3 months after feeding and bite damage of leaves were recorded.

(e) Evaluation of the allelopathic effect on plant by MF-1

The antagonistic effects or harmful effects on neighboring plants in plant community derived from the transgenic plants are considered to be a critical issue. The allelopathic impact assay was used in this biosafety assessments to assay the allelopathic impact of the leachates released from dried leaves (Fujii *et al.*, 2003, 2004; Oguchi *et al.*, 2014). The sandwich method was employed to test whether MF-1 released any metabolites to damage surrounding plants in a vegetation. By monitoring the growth and germination rate of lettuce seeds, data from sandwich method revealed that no significant difference between non-transgenic orchid, HA, and transgenic orchid, MF-1 in the seedling growth and germination rate (Fig. 13).

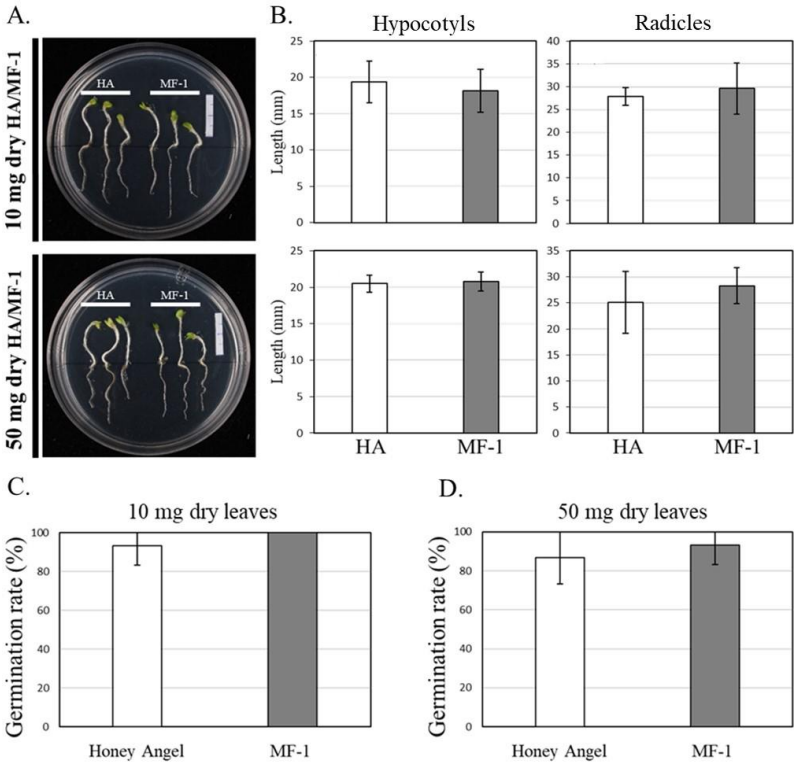


Fig. 13 An allelopathic evaluation using the sandwich method to assay the repression effect of MF-1. The growth rate of (B) lettuce hypocotyl and radicle, and (C-D)germination rate were evaluated under 10mg or 50mg dry leaves treatment. Dark grey bar represents the using of MF-1, light bar represents HA. The data were presented average of three replicates ± SD for each, respectively. It did not show any significant difference between MF-1 and HA.

D. Possibility of gene flow to animal/plant/pathogens and its potential threats to environment

There are two possible pathways that gene may flow from transgenic orchids into animals, plants or any pathogens.

One is by the way of the contaminants of transgenic orchid. Because gene transformation process is mediated by *Agrobacterium tumefaciens*. The bacteria may contaminate and adhere to the transgenic plants and be released out to environmental living organisms. The second pathway is the release to environments by surrounding rhizospheric microbial organisms. Aiming at these two possibilities, we first tested the transgenic plants for the adhered Agrobacterium cells.

(a) Assay on the bacterial contamination of *Agrobacterium* microorganisms adhered in MF-1 seedlings

This experiment was based on National Institute for Agro - Environmental Sciences (Ibaraki, Japan) study for genetic characteristics and biosafety assessment of genetically transgenic anti-TMV tomatoes transgenic with *Agrobacteria*. (組換えトマト安全性評価研究グループ，1992) The leaves and roots were detached and placed on 1/2 MS medium containing kanamycin (200 ppm), and incubated in room temperature for one week, dark condition. After one week, there were no microbial colony found in both incubations of transgenic orchids (MF-1) and recipient orchid (HA) (Fig. 14). It indicated that the working process from screening to growing transgenic oncidium orchids was conducted very strictly to keep transgenic orchid plants from the contamination of *Agrobacterium* residue in the outside environment.

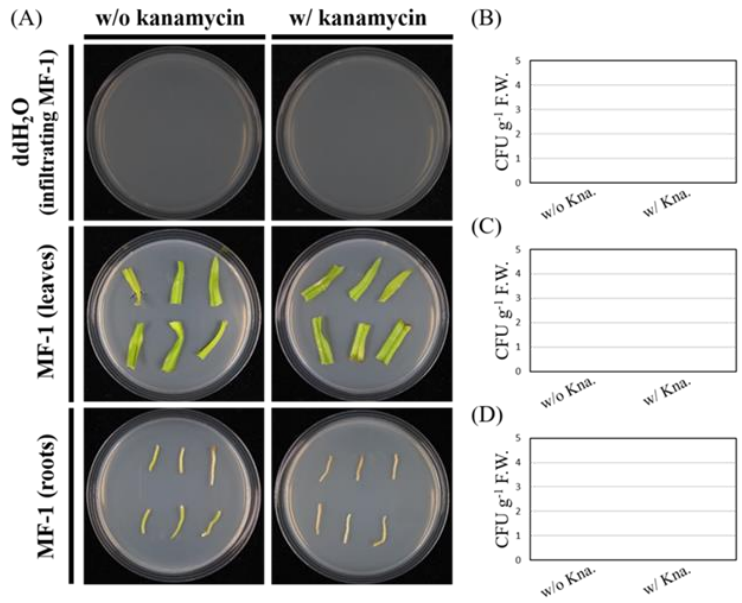


Fig. 14 Assay on *Agrobacterium* residue adherence to MF-1 seedlings. (A) The MF-1 whole plant tissue culture seedlings were immersed in 30 ml of ddH_2O for 10 minutes, and 5 ml of the soaking solution was applied to the plate, and the leaves and roots of the MF-1 were placed on the YEB solid medium at 28℃ for one week. The number of colonies were calculated in terms of fresh weight per gram of fresh weight (F.W.). (B) ddH_2O; (C)leaves; (D)roots; The data were the average of three replicates ± SD. The composition of YEB medium: 5g/l beef extract, 1 g/l yeast extract, 5g/l peptone, 5g/l sucrose, 0.5g/l $MgSO_4$, 1.5% w/v agar.

(b) Detection of the possibility of gene flow into the rhizospheric microbial organisms

To test whether gene flow from transgenic orchid into the rhizospheric microbial cells, the growing root and substrates were wash out off by using sterilized water. The flute water was collected and spread on agar plates and incubate under ambient temperature. As shown in Fig. 15, the growing colonies were picked up and extracted for the genomic DNA. By using PCR detection for *HPTII* gene, primer pairs 5’-GATGTAGGAGGGCGTGGATA-3’ and 5’-CGTCTGCTGCTCCATACAAG-3’ were employed for amplification. The results showed that the presence of *HPTII* gene was not detected in the rhizosphere of HA and MF-1, indicating that there was no outflow of *HPTII* gene in MF-1, so it did not affect the changes of soil microbial phase, as shown in Fig. 16, 17.

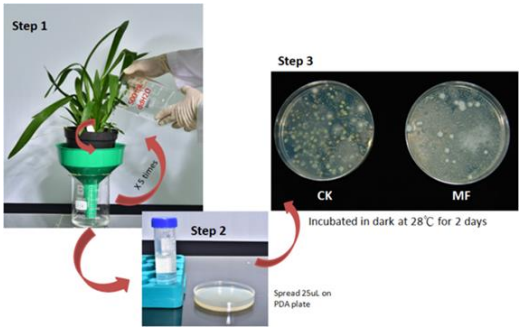


Fig. 15 Cultivation of the rhizospheric microorganisms by collecting the root eluates of oncidium orchids. By using ddH_2O to rinse the orchid root tissues, the root eluates were collected and cultivated in agar medium for monitoring the rhizopheric microorganisms. CK: HA; MF: MF-1.

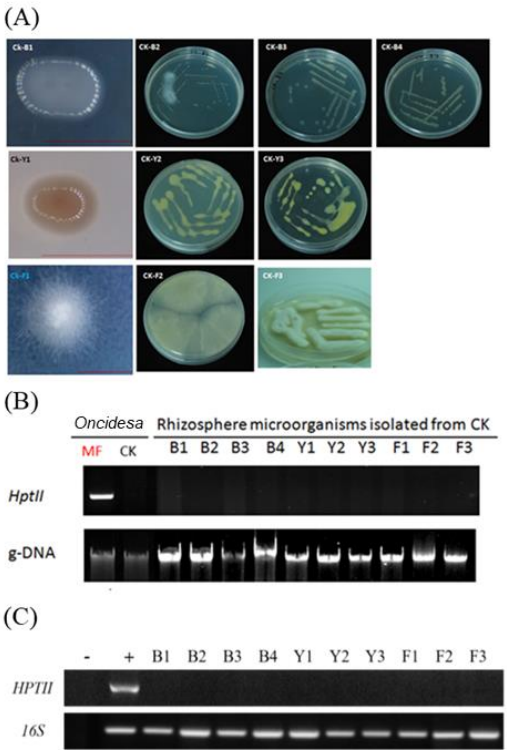


Fig. 16 Detection of *HPTII* gene in the rhizospheric microbial cells from root eluates of HA (CK) by using PCR. (A) Microbial cultures prepared from the root eluate water of HA. (B) Assay of *HPTII* gene integration by genomic PCR from 10 microbial cultures prepared from root eluate of HA, showing no gene integration in microbial cell. (C) Assay of *HPTII* gene integration by RT- PCR from 10 microbial cultures prepared from root eluate of HA, showing no *HPTII* gene integration. Each colony of ten different microbial cultures was picked and cultured in broth and extracted for genomic DNA. Genomic DNA were amplified for assaying *HPTII* gene following the standard molecular tool. (-: negative control, +: positive control).

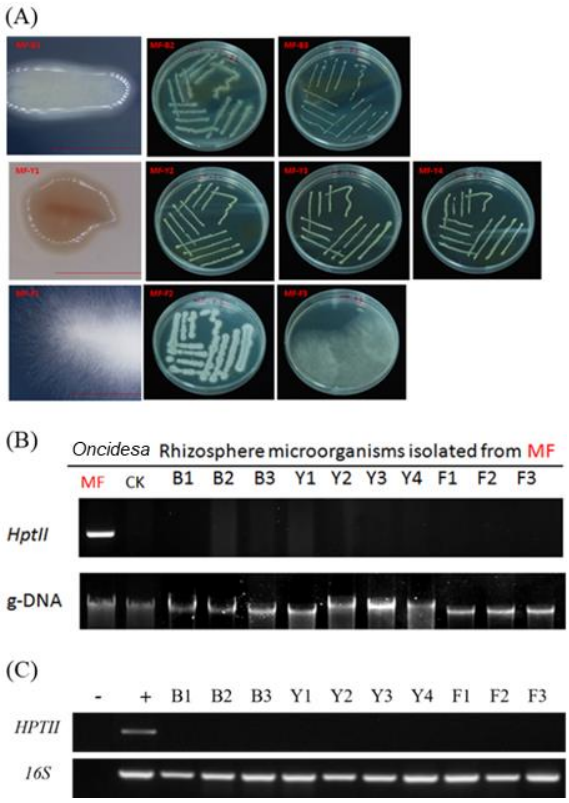


Fig. 17 Detection of *HPTII* gene flowing into the microbial cells in the rhizosphere of MF-1 by using PCR. (A) Microbial cultures prepared from the root eluate water of MF-1. (B) Assay of *HPTII* gene integration by genomic PCR from 10 microbial cultures prepared from root eluate of MF-1, showing no gene integration in microbial cell. (C) Assay of *HPTII* gene integration by RT- PCR from 10 microbial cultures prepared from root eluate of MF-1, showing no *HPTII* gene integration. Each colony of ten different microbial cultures was picked and cultured in broth and extracted for genomic DNA. Genomic DNA were amplified for assaying *HPTII* gene following the standard molecular tool. (-: negative control, +: positive control).

E. Potential risks by the gene flow into environment

The gene flow and gene contamination are the subjects that affect the biodiversity and the most concerned issues in transgenic industry. For outcrossing crops or anemophilous crops, the doubts on gene drift may be quite significant (Wang *et al*., 2004). The potential risk has been evaluated and reported. Back to our present topic, MF-1 has been tested in various experiments for the biodiversity danger. In conclusion, it basically has pollen infertile, reproductive sterility, and the pollen formation is very abnormal; therefore there is no risk on the pollen transfer. As oncidum orchid is native to Central and South America, there are no such native species in Taiwan. In addition, our allelopathic test has proven that the gene flowing into living organisms neighboring surrounding environment very difficult. Taken together, the assumption that the genes in MF-1 might flow to other organisms is not feasible.

(5) Treatments of the used plants after the experiments

All the transgenic orchid plants after the experimental tests and the dead plant tissues incurred in the testing period were treated as follows:

A. Plants were separated from the substrates (pea moss, tiny stones and wood pieces, etc.).
B. Packing plants, substrates, pot in the PE plastic bag and autoclaved with 125℃, 20 kg/cm^2 for 40 min
C. Discarding as the same as general waste (Fig. 18).

Fig. 18 Process flow of the treatment of the used plants after the experimental test. Whole plants and debris and pot substrates were autoclaved at 125℃, 20 kg/cm^2 for 40min after experimental test.

(6) Conclusions

A. Prof. Yeh, Kai-Wun, Institute of Plant Biology, National Taiwan University successfully employed RNAi technology to suppress the endogenous '*phytoene synthase*' in the petal tissue and successfully modified *Oncidesa* Gower Ramsey 'Honey Snow' MF-1 with white hue florets. The evaluation on genetic traits of transgenic *Oncidesa* Gower Ramsey 'Honey Snow' MF-1 and environmental biosafety assessment were conducted at Academia Sinica, Tainan. There are similar physiological/morphological found between two varieties, except the flower pigment is different with yellow in HA, but white in MF-1 (Fig.1). The shelf life of both post-harvested flowers also showed no significant difference.

B. Cross hybridization test by intra-species, inter-species and inter-genus further confirmed that transgenic *Oncidesa* Gower Ramsey 'Honey Snow' MF-1 failed to form fruit pod normally. Based on microscopic observations, the pollen sterility was affirmed by basic cytological assays to dissect the meiosis process and revealed that abnormal chromosomal pairing and segregation were present. The shape of the pollen grains was irregular and size varied, compared to the uniform appearance in wild orchid of *Oncidium sphacelatum*. The cytological evidences also support that MF-1 was almost sterile and difficult to pollinate.

C. Designing white-flowered oncidium variety by blocking the carotenoid biosynthesis pathway in the yellow-coloured flowers of an Oncidium hybrid, such as *Oncidesa* Gower Ramsey 'Honey Snow' MF-1 using RNAi-technology, was effective and promising. Comparing to traditional method using overexpression of promoter, floral specific promoter *pCHRC* (Chromoplast specific carotenoid associated gene) was employed to specifically express in petal tissue (Chiou *et al.*, 2008). Successful silencing of Pchrc promoter specifically in petal tissue was obtained by a small fragment of its own phytoene synthase cDNA of 500 bp in length to achieve white orchid transgenic varieties. In addition, HPTII was used as a marker, suggesting that to be non-toxic to health of animals and microorganisms (Xu *et al.*, 2009; Zhuo *et al.*, 2009). Overall, obtaining MF-1 by RNAi technology was relatively to be safe to the environment and to the feeders.

D. The gene flow and gene contamination are the subjects that affect the biodiversity and the most concerned issue in transgenic industry. MF-1 is self-incompatible, which is basically pollen infertile and reproductive sterile, and the pollen formation is very abnormal. The hybridized with other oncidium was also incompatible; therefore there is low risk of a pollen transfer.

E. The risk of becoming a weed in the environment is one of the concerns on all transgenic plants. Pollens of MF-1 are sterile. Even the sparse pollination is not able to disperse in the environment. Moreover, oncidium orchid plants are epiphytic with sympodial growth habitat, so there is no rhizome organ to propagate its seedling. Furthermore, oncidium orchid have been cultivated in Taiwan for more than 30 years in confined environment, thus requiring a specifically designed environment to grow. Taken together, the weediness is very difficult.

F. After assessing the direct or indirect effects of gene transfer on the non-target organisms, the results indicated that the possibility of *HPTII* gene horizontally transferring to snails, slugs, fruit fly and rhizosphere microbial flora was low and also confirmed its non-toxic to health of them. The allelopathic assay revealed that no harmful/ antagonistic effect against the surrounding plants. Taken together, MF-1 is a relatively safe genetically modified plants to the ecological environment.

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(7) References

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