Plant Pathology Bulletin 14:159-176, 2005

論　說

無篩選標示基因 (marker-free) 轉基因植物之構築及其最新發展

林靜宜[1]　詹富智[1,2]

[1] 國立中興大學植物病理學系
[2] 聯絡作者，電子郵件：fjjan@nchu.edu.tw ; 傳真: +886-4-22854145
接受日期：中華民國 94 年 8 月 25 日

摘　要

林靜宜、詹富智. 2005. 無篩選標示基因 (marker-free) 轉基因植物之構築及其最新發展. 植病會刊 14: 159-176.

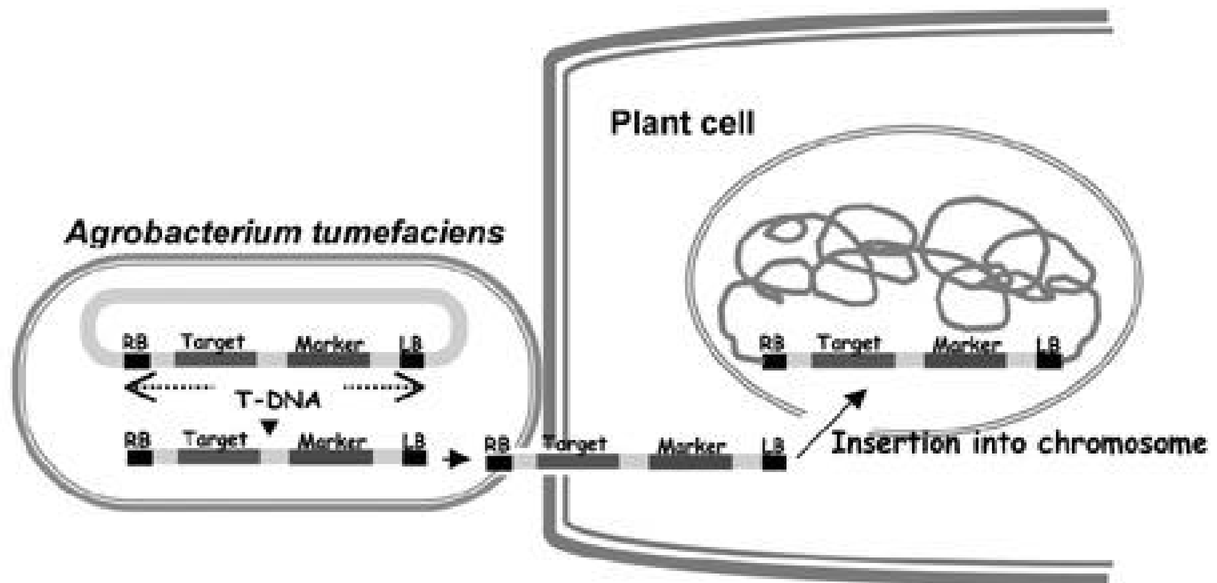
植物基因轉殖系統需藉篩選標示基因 (selection marker genes) 以有效率地篩選出轉殖細胞或植物。大多數篩選標示基因皆是利用其具有抗生素抗性或殺草劑抗性來進行篩選。篩選標示基因轉殖後仍存在於植物中，引發大眾對環境安全及人體健康影響之疑慮。故將篩選標示基因自轉基因作物移除，已成當前趨勢。目前已有數種產生無篩選標示基因之轉基因植物的系統被建立，包括共轉型法 (co-transformation)、特定位置重組法 (site-specific recombination)、跳躍子系統 (transposition)、同源重組 (homologous recombination) 及正向篩選法 (positive selection) 等。經由這些系統所產生之無篩選標示基因 (marker-free) 作物將可大幅地提升一般民眾對於轉基因作物的接受度。本文即針對現今不同之無篩選標示基因植物轉殖系統之策略作完整之彙整並討論其優缺點及未來發展趨勢。

關鍵詞：篩選標示基因、無篩選標示基因、轉基因植物、葉綠體轉殖、農桿菌轉殖

緒　言

自 1984 年轉基因植物 (菸草) 被研發成功後[53]，植物基因轉殖技術已廣泛地應用於將外源基因導入植物的基因體內並可打破植物物種間的界定，達到修飾或改造植物特性的目的。近二十年來成功地發展出許多具有商業價值之轉基因作物，例如：玉米、大豆、棉花、馬鈴薯及番茄等作物，對農業上的應用包括抗病品種及其他品種的改良、食品品質及人體健康等方面提供了實質且重大的改善。植物基因轉殖技術之流程主要包含下列三步驟: (1) 將外源基因導入植物基因體內, (2) 轉基因細胞或植物之篩選, (3) 轉基因植物再生成完整植株。至今已發展出多種技術，例如：電孔法 (electroporation)、微注射法 (microinjection)、花粉管法 (pollen tube pathway)、聚乙烯二醇法 (polyethylene glycol, PEG)、基因槍法 (particle bombardment) 及農桿菌轉殖法 (*Agrobacterium*-mediated transformation) 等，可將外來基因導入植物細胞中[49]，其中以農桿菌轉殖法及基因槍法最被廣泛應用。

如何篩選出已轉殖成功之細胞或轉基因植物為植物基因轉殖過程中非常重要的一環，最常使用的方法是將適當的篩選標示基因 (selectable marker gene) 連同所欲轉殖的基因同時送入植物細胞內，以便篩選出轉殖株 (圖一)。篩選標示基因依其性質可分為三大類，分別為抗生素抗性基因、殺草劑抗性基因及生長代謝抑制基因[75]；其中具抗生素抗性或殺草劑抗性的抗性基因是目前最常使用的篩選標示基因，可使轉基因細胞對添加於培養基中的抗生素或殺草劑具有抗性，而非轉基因細胞則會被殺死。因此，帶有轉基因的單細胞即可自大量的非轉基因細胞中被篩選出來。然而，這些篩選標示基因經轉殖之後仍會留存於轉基因植物中且在植物內不具任何實質上的功能。目前已知當非轉



圖一、農桿菌轉殖法。位於兩 border 之間之 DNA 片段稱為 T-DNA，T-DNA 自兩 border 之間被剪切後可被送入植物細胞中並嵌入植物之基因組內。

Fig. 1. Schemes for *Agrobacterium*-mediated transformation. T-DNA which locates between right border (RB) and left border (LB) is transferred into plant cell and integrated into plant genome.

基因細胞於含有這一類化合物之選擇性培養基中無法生長或是死亡時會對轉基因細胞產生負面的效應，因為死亡的細胞會抑制養分供給至轉基因細胞甚至是分泌有毒的物質，進而降低轉基因細胞增殖及分化成為轉基因植物的能力[27]。另外，目前供篩選標示基因使用之啟動子 (promoter) 常侷限於特定的種類，若重複地利用這些啟動子易導致基因沉寂作用 (gene silencing) 的產生；再者，有效且適用的篩選標示基因種類亦有限[45]。

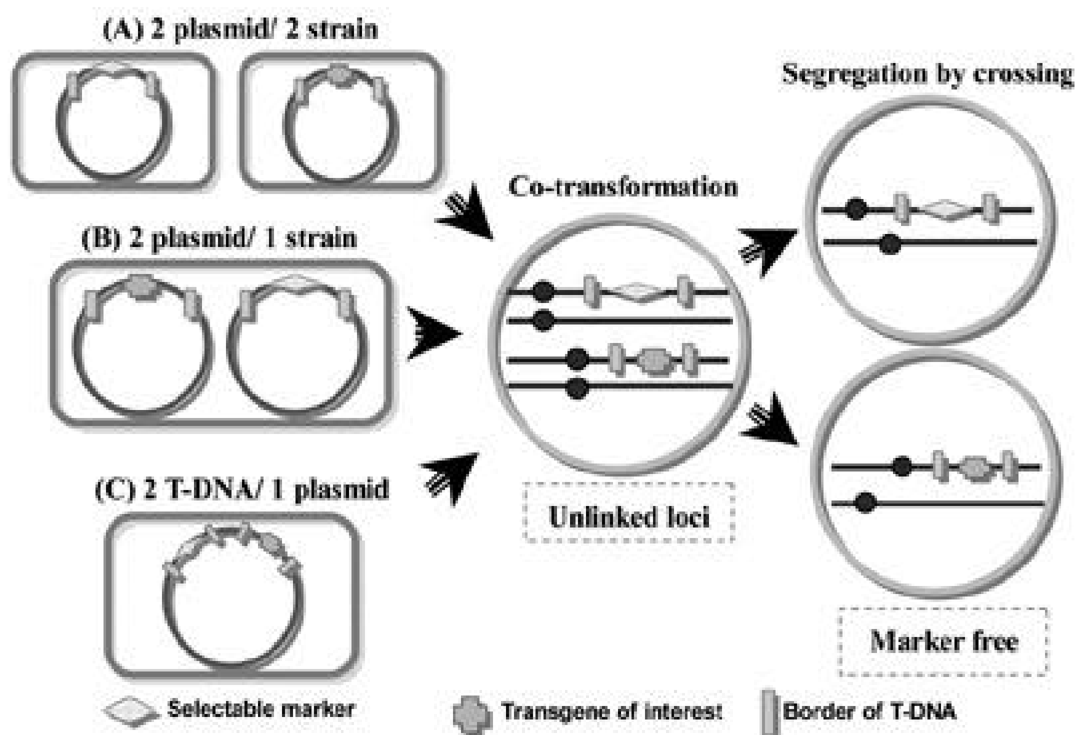
除此之外，應用於轉基因作物中的篩選標示基因之安全性近年來已引發大眾的疑慮，其潛在的隱憂主要包括二大類[6, 58, 105, 109]，一是基因改造作物對生態環境平衡之衝擊，二為基因改造作物作為食品及飼料用途時對人體及其他生物健康之影響。例如殺草劑抗性基因可能會藉著花粉傳播至野生植物及與轉殖作物具親源性之雜草上，使其它野生植物變成為害作物的頑強雜草 (super weed) 並導致基因污染 (genetic pollution)[12, 58]。抗生素抗性基因可能將其抗性間接或直接的轉移至人體內的微生物、腸胃中的生物、土壤中的菌類或是食用這些轉基因植物的動物腸道內[23, 35, 47, 75, 77, 79]，而改變原有的微生物相。雖然這些議題尚缺乏強而有力的證據來佐證[47, 79, 84]，但是英國於「發展基因改造作物之最佳政策」方針中仍建議基因改造作物使用之外來基因應侷限在最少量[85]；2001年之歐盟標準規範法令 (European Council Directive 2001/18/EC) 關於「釋放至環境中之基因改造生物的安全性」中要求於 2004 年即「淘汰」以醫學臨床上相關之抗生素抗性基因作為篩選標示基因使用至基因改造生物內[85]。而處理關於基因改造問題一向極為審慎的歐盟，已自 2005 年起禁止以抗生素抗性基因作為篩選標示基因之轉基因作物輸入；更決議於 2008 年後不允許實驗室所生產之轉基因試驗植物含有抗生素抗性基因作為篩選標示基因。因此，植物基因轉殖工程技術朝向發展無篩選標示基因 (marker-free) 之轉基因植物已成為當前重要之研究指標。此外，無篩選標示基因的策略亦可應用至生產多基因之轉殖植物而不受限於供目前使用之篩選標示基因種類[121]。目前已有多種產生無篩選標示基因轉基因植物之系統被發展建立，包括利用移除 (removal) 的策略去除篩選標示基因的共轉型法 (co-transformation)、特定位置重組法 (site-specific recombination)、跳躍子系統 (transposition)、同源重組法 (homologous recombination) 及利用與生長代謝等相關基因作為篩選標示基因以取代常用之抗生素抗性及殺草劑抗性基因的正向篩選法 (positive selection)。本文即針對現今應用於植物轉殖系統之無篩選標示基因轉殖策略作完整之彙整。

共轉型法 (Co-transformation)

農桿菌轉殖法是利用農桿菌感染時具有將其 Ti- 質體 (tumor-inducing plasmid, Ti-plasmid) 中位於兩 border (right border, RB 及 left border, LB) 之間一段稱為 T-

DNA (transferred DNA) 的序列嵌入寄主基因體中的特性，藉由將標的基因及篩選標示基因構築至同一段 T-DNA 中，此兩基因就可同時被送入植物細胞內 (圖一)。因兩者皆位於同一 T-DNA，故會插入植物基因體中連鎖的基因座 (linked loci)，篩選標示基因將一直存留於植物中而無法與標的基因分離。因此我們可以將篩選標示基因與欲轉殖之標的基因分別構築於不同的 T-DNA 上，其中一組攜帶有篩選標示基因，另一組則攜帶所欲轉殖之標的基因片段，將此二組不同之 T-DNAs 送入同一個植物細胞內，則位於不同 T-DNA 上之篩選標示基因及欲轉殖之標的基因片段即有機會分別嵌入植物染色體中不連鎖的基因座 (unlinked loci)，就可藉親本自交所產生之子代分離得到無篩選標示基因之轉基因植物 (圖二)，共轉型法之發展即植基於此一概念[27]，此法又可依構築的形態細分為：(1) 兩組 T-DNA 構築於二個不同的植物轉殖載體上並分別送入不同的農桿菌中後，將此二農桿菌混合進行植物轉殖之雙載體/雙菌株法 (two-plasmid/two-strain) (圖二A) [18, 20, 21, 64, 74]，(2) 兩組 T-DNA 構築於二個不同之植物轉殖載體但送入同一個農桿菌中進行轉殖之雙載體 /單菌株法 (two-plasmid/one-strain) (圖二B) [13, 19, 57]，(3) 兩組 T-DNA 構築於單一植物轉殖載體上之雙 T-DNA/ 單載體法 (two-T-DNA/one-plasmid) (圖二C)[21, 64, 69, 117](表一)。

由於共轉型法操作簡易並可順利得到不具篩選標示基因之轉殖植物，為目前使用最廣泛的方法。然而有報告指出此三種共轉型法具有廣泛的變異性，亦很難於不同的方法之間提出一個有系統性的評論[84]。例如 Komari 等人[64]以 neomycin phosphotransferase II gene (*npt* II) 或 hygromycin phosphotransferase gene (*hpt*) 作為篩選標示基因、glucuronidase gene (GUS) 為標的基因，分別利用雙載體/雙菌株法及雙 T-DNA/ 單載體法在菸草 (*Nicotiana tabacum*) 及水稻 (*Oryza sativa*) 上試驗，發現採用雙載體/雙菌株法於兩次重複實驗中，經初次抗生素篩選之後亦具有 GUS 活性的轉殖比率分別為 22%, 35% (菸草) 及 2%, 14% (水稻)，經自交分離後不具篩選標示基因 (*npt* II 或 *hpt*) 而僅具 GUS 活性之子



圖二、以共轉型法產生無篩選標示基因轉基因植物。(A) 雙載體/雙菌株法、(B) 雙載體/單菌株法、(C) 雙 T-DNA/單載體法。其原理是藉農桿菌轉殖法將二組不同之 T-DNAs 送入同一個植物細胞內，其中一組攜帶有篩選標示基因，另一組則攜帶所欲轉殖的基因片段。若位於不同 T-DNA 上之篩選標示基因及欲轉殖的基因片段分別嵌入植物染色體中不連鎖的基因座，則可藉親本自交所產生之子代分離得到無篩選標示基因之轉基因植物。

Fig. 2. Schemes for generating marker-free transgenic plants by co-transformation. Three approaches of co-transformation system include: (A) 2 plasmids/2-strains, (B) 2 plasmids/1 strain and (C) 2 T-DNAs/1 plasmid. A selection marker gene and a gene of interest from two different T-DNAs are integrated into unlinked loci by co-transformation. The gene of interest is then segregated from the selectable marker gene by sexual crossing. (modified from Ref. 27).

表一、產生無篩選標示基因轉基因植物之方法

Table 1. Methods used to generate marker-free transgenic plants

Methods	Crops	References
Co-transformation		
Two plasmids/ two strains	Tobacco	20, 21, 64, 74
	Rice	64
	Brassica napus	18
Two plasmids/ one strain	Tobacco	13, 19, 57
	Rapeseed	13
(dual binary vector: pGreen/ pSoup)	Rice	1, 2, 104
	Wheat	83
Two T-DNA/ one plasmids	Tobacco	8, 21, 64, 117
	Rice	64, 69
	Soybean	110
	Barley	72, 111
	Maize	76
P-DNA	Potato	88
Site-specific recombination		
Cre/*loxP*	Tobacco	11, 38, 90
	Arabidopsis	90
(heat shock induce promoter)		51
(chemical-regulated)		120
	Maize	115
	Rice	50
(chemical-regulated)		97
FLP/*frt*	*Arabidopsis*	70
	Maize	71
R/*RS*	Strawberry	91
Phage-attachment region (*attP*)	Tobacco	118
2 site-specific (Opinion)	-	98
Multi auto transformation (MAT)		
ipt-type MAT	Tobacco	25, 28, 101
	Rice	33
rol-type MAT	Tobacco	29
GST-MAT	Tobacco	32
	Maize	100
Transposon		
Ac/Ds transposon system	Tomato	40
(Transposon-induced)		113
(marker gene on the mobile element)	Tobacco	28, 29
(marker gene on the mobile element)		41
	Rice	10, 59

代比例則分別為 71% (菸草) 及 100% (水稻)；若利用雙 T-DNA/ 單載體法轉殖菸草及水稻則同時具抗生素抗性及 GUS 活性之轉殖植物比例分別為 50% (菸草) 及 52% (水稻)，其僅具 GUS 活性之子代則為 56%,100% (菸草) 及 65% (水稻)，其轉殖效率會因採用方法及作物不同而異[64]。其可能原因如使用之農桿菌種、植物種類及其生長狀況、植物細胞和農桿菌之間的交互作用及植物細胞對 T-DNA 之接受度等等來自農桿菌、植物及環境之因子皆為影響轉殖效率之變數，但是整體而言利用共轉型法所得不含篩選標示基因之效率是高於分別進行二次的單一轉型方式[27]。除了採用不同的共轉型法其效率具差異性之外，農桿菌合成之篩選標示基因數目多寡亦會影響轉殖效率，Jacob and Veluthambi[57]分別以具高套數 (10-15 copy number) 之二位元載體攜帶 *npt* II 基因或是低套數 (1 copy number) 之共整合載體 (cointegrate vector) 攜帶 *hph* 基因作為篩選標示基因，並採用雙載體/單菌株法作為轉殖策略研究發現，若以低套數之載體攜帶篩選標示基因 (*hph*) 則其獲得無篩選標示基因植物之轉殖效率較佳。

共轉型法的主要缺點是嵌入植物染色體中不連鎖

基因座之轉基因需依賴自交的方式於子代中分離後，才能獲得無篩選標示基因之轉基因植物，因此需篩選大量的轉殖植物，不僅時間成本龐大且不適用於生長緩慢之木本植物上。故近來有學者將綠螢光蛋白基因 (green fluorescent protein gene, GFP) 和 *npt* II 基因構築於同一 T-DNA，而標的基因 (GUS) 則放入另一 T-DNA 利用雙 T-DNA/ 單載體法進行轉殖，即可透過發出螢光與否篩選出無篩選標示基因轉基因植物，進而節省時間及降低篩選藥品的消耗量[8]。

農桿菌轉殖法最大的特點即是將位於 RB 及 LB 之間的 T-DNA 送入植物細胞內，但已有研究指出除了 T-DNA 區域之外，轉殖過程中亦常會將載體其他部份之序列 (稱為 backbone DNA) 一起送入植物細胞，而實際上除了欲轉殖基因之外，其他序列對植物而言都是不必要的[87, 107]。因此近來更進一步發展出降低非植物本身來源之序列 (如backbone DNA) 嵌入植物染色體中的新策略。Rommens 等人[88]於水稻及阿拉伯芥 (*Arabidopsis thaliana*) 基因體序列中比對出與農桿菌之 RB 及 LB 具高度相似性之序列 (border-like sequence)，藉此自馬鈴薯中選殖出和農桿菌的 RB 及 LB 相似之 DNA 片段，構築此一由植物序列組成之 P-DNA (plant-derived DNA fragment) 用以替代傳統農桿菌之 T-DNA 來攜帶外源基因，藉此即可經由共轉殖及子代分離的方式獲得除外源基因外完全皆由植物 DNA (native DNA) 而來之無篩選標示基因轉基因植物，大大的降低外來 DNA 序列於植物中的含量[87, 88]。

特定位置重組法 (Site-specific recombination)

某些微生物中具有特定位置重組酶 (site-specific recombinase) 可辨識特定的正向重複 (direct repeats) 序列，並切下位於此辨識序列 (recognition site) 之間的區域，因此常被應用在高等真核生物的基因工程技術中[85]。此特性約十五年前即被應用至植物轉殖技術上，藉由將供細菌噬菌體 P1 之重組酶 (Cre) 辨識之特定正向重複序列 (*lox*P) 置於篩選標示基因的兩端，如此一來即可藉重組酶的作用將篩選標示基因自植物基因體中移除[11] (圖三)。至今已有一系列的特定位置重組系統被建立包括:細菌噬菌體 P1 (bacteriophage P1) 之 Cre/*lox* 重組系統[11, 38, 51, 90, 120]、酵母菌 *Sachromyces cerevisae* 之 FLP/*frt* 系統[16, 37, 62, 70, 71]及 *Zygosaccharomyces rouxii* 之 R-*RS* 系統[25, 81, 100, 101]。

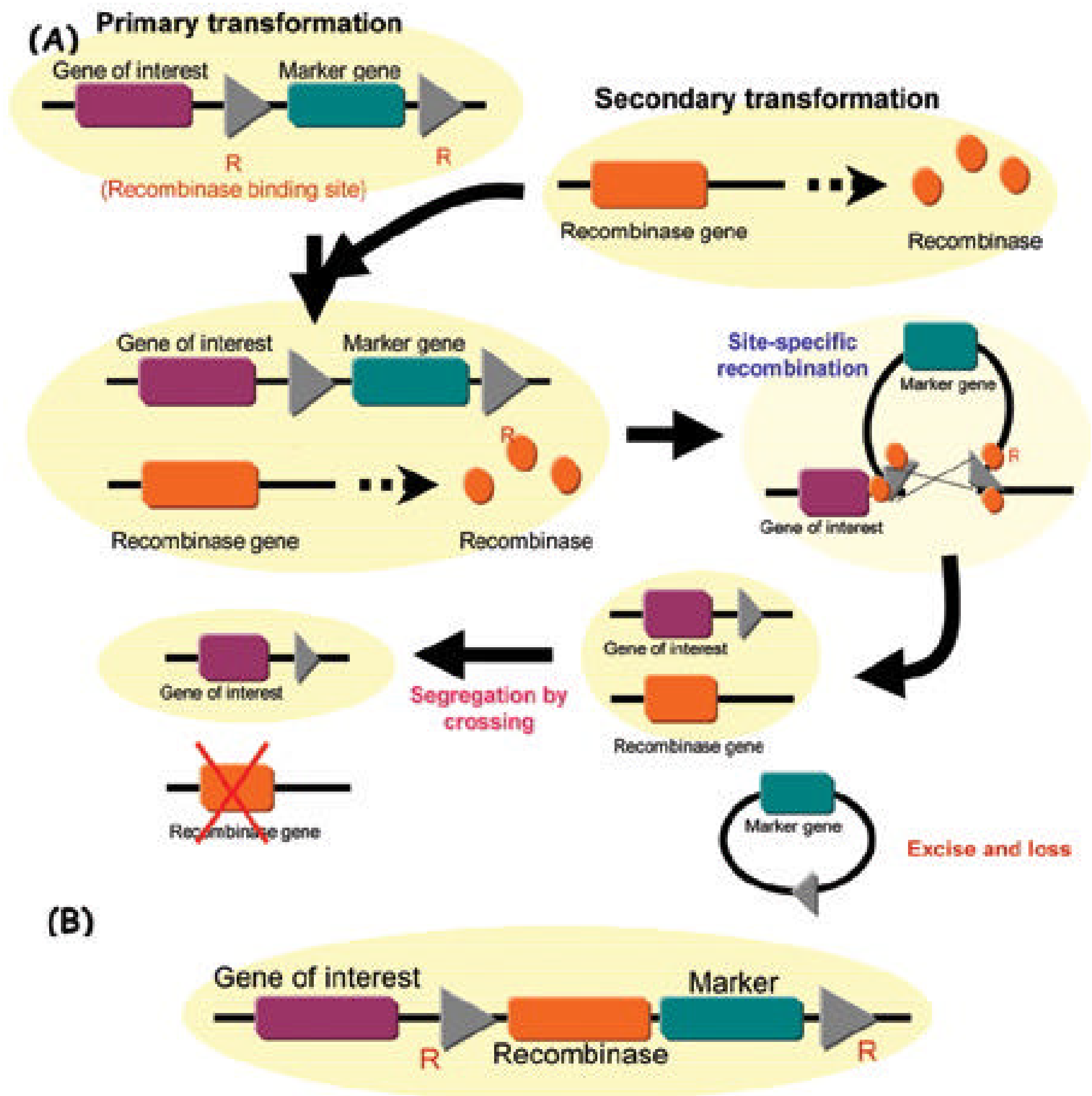
早期的特定位置重組法須將重組酶及供其辨識之正向重複序列分別經兩次的轉型或植物雜交的方式送入同一植物細胞中，以利移除篩選標示基因的進行，然而重組酶往往仍殘存於植物細胞中，因此與共轉型法相同，需經子代分離後方能獲得較完整之無篩選標示基因轉基因植物[11] (圖三A)。有鑑於此，若在同一載體上將重組酶與欲移除之基因一同置於正向重複序列之間即可解決需子代分離之困擾 (圖三B)。

為了更進一步使重組酶在轉殖後於適當時機再行表現以提高轉殖效率，乃發展利用誘發性特定位置重組 (inducible site-specific recombination) 系統來移除篩選標示基因[120]，其原理是利用賀爾蒙 β-雌二醇 (β-estradiol) 配合啟動 XVE 誘發系統 (transactivator XVE)[119, 120]來調控 cre-recombinase 基因的表現，以提高移除篩選標示基因效率之目的。此外，最近另有一套改良式的系統被建立，結合誘發性特定位置重組法及雙功能之篩選標示基因 (*Escherichia coli* cytosine deaminase-Neomycin phosphotransferase II gene, *cod*A-*npt*II) 的使用；首先利用 nptII 基因作為正向篩選接著利用化學誘導性之 codA 基因再行負向篩選出真正的無篩選標示基因轉基因植物[91]，此一系統的策略較其他系統省時且重組酶亦可隨篩選標示基因一起被移除。近來更有學者提出雙特定位置重組法 (two site-specific recombination) 的概念，將兩載體藉轉殖或雜交後分別送入植物細胞內，因其中一載體攜帶外源基因、篩選基因及兩組正向重複序列，另一載體則攜帶兩組重組酶，如此一來就可藉其中一組重組酶系統經同源交換的方式將兩載體攜帶之基因整合至相鄰的位置，此時另一組重組酶系統則可進行特定位置重組將篩選標示基因移除而產生無篩選標示基因轉基因植物[98]。

除上述之重組酶系統具有將特定位置之序列移除的功能之外，另有利用同源重組 (homologous recombination) 的方法將特定區域之序列移除[86]。其原理與特定位置重組法相似，乃將位於兩正向重複序列之間的區域切除。早期於體細胞 (somatic cells) 中即有學者利用同源重組的方法切除序列，但其效果不佳[86]，因而被認為不適合作為移除篩選標示基因的系統。直至 Zubko 等人[118]利用源自細菌噬菌體之 phage attachment site (*attP*) 扮演正向重複序列的角色，藉由染色體之間的重組 (intrachromosomal recombination) 成功地將篩選標示基因移除[118]，不僅較以往之同源重組法具有更佳的效率，且可免除重複轉殖或是子代分離之步驟而縮短所需時程。同源重組法於質體轉殖技術中扮演非常重要的角色，因此同源重組法亦可應用於葉綠體轉殖技術中作為移除篩選標示基因的媒介，近來已有相關文獻證實其可行性[55] (將於稍後作詳述)。

跳躍子系統 (Transposition)

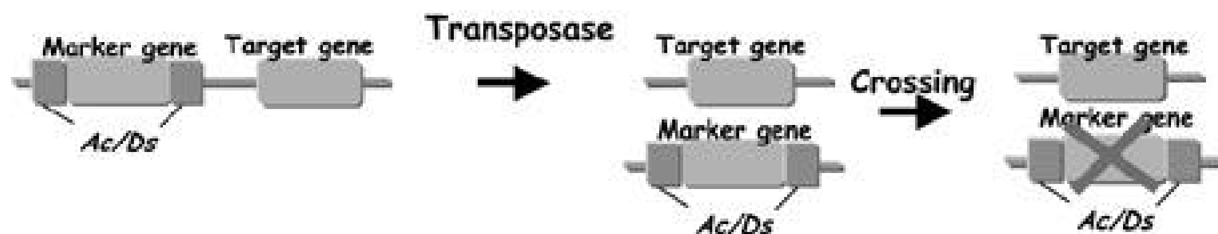
跳躍子 (transposon or transposable element) 又稱轉



圖三、(A)以特定位置重組法移除篩選標示基因之基本原理。將篩選標示基因 置於兩組供重組瓷辨識之正向重複序列 (R) 之間，一但重組瓷開始表現就可將篩選標示基因自植物基因體中於轉殖篩選的過程中移除移除。(B)改良式特定位置重組法。將篩選標示及重組瓷置於兩供辨識之正向重複序列 (R) 之間，即可將篩選標示基因及重組瓷同時於篩選的過程中移除。

Fig. 3. (A) Basic strategy for the excision of selection marker genes by site-specific recombination. The selection marker gene is inserted into the transformation vector between two recombinase biding sites (R) that are recognized by a recombinase. After expression of recombinase, the marker gene is excised from the plant genome. (B) Modified strategy for the excision of selection marker genes by site-specific recombination. The selection marker gene and recombinase are inserted into the transformation vector between two recombinase biding sites (R) that are recognized by a recombinase. The marker gene and recombinase gene is excised from the plant genome without crossing.

位子，是一段具有可轉移位置特性之基因序列，最早於 1947 年在玉米中被發現，目前以 *Ac/Ds* 系統最為常見，*Ac/Ds* 系統可分為兩種，一為自動 (autonomous) 跳躍子 (如*Ac*)，意指跳躍子含有轉位瓷 (transposase) 可辨識跳躍子兩端特殊之序列以進行切割，之後再將位於跳躍子兩端間的序列插入植物基因體中的其他位置 (cut-and-paste)；另一為被動 (non-autonomous) 跳躍子 (如*Ds*) 即指跳躍子之間不含有轉位瓷或是具無功能之轉位瓷，因此需要提供正常之轉位瓷才能發揮轉移的特性[36, 46]。由於其原理與特定位置重組法相似，因此亦



圖四、以跳躍子系統 (Transposition) 移除篩選標示基因之圖示。將轉位子序列 (*Ac/Ds*) 置於篩選標示基因之兩端，當轉位*表現時可將篩選標示基因切下並轉移至染色體其他位置後即可藉親本自交所產生之子代分離得到無篩選標示基因之轉基因植物。

Fig. 4. Scheme for generate marker-free transgenic plants by transposition. The marker gene is flanking with the transposable element sequence (*Ac/Ds*). The activation of transposase allows the relocation of the selection marker gene to new chromosome positions. The target gene is then segregated from the selectable marker gene by sexual crossing (modified from Ref. 52).

具有應用於移除篩選標示基因的潛力 (表一)。一般是將篩選標示基因置於跳躍子之間，而此跳躍子可於移位 (transposition) 後移除 (圖四)[41]。跳躍子系統 (*Ac/Ds*) 首先成功的應用於番茄上來達到分離篩選標示基因及欲轉殖之基因的目的 [40, 113]。但使用跳躍子系統 (*ipt* gene/*Ac* element) 至菸草及山楊屬之植物上時，所得到之無篩選標示基因轉殖效率卻不高 [28, 29]。跳躍子系統除了可將篩選標示基因及欲轉殖之基因分離而嵌入植物基因體中不連鎖的位置之外，也可獲得一系列具有不同嵌入位置之轉基因植物。然而，仍需進行子代分離才能得到無篩選標示基因的轉基因植物，因此亦是相當耗費時間[84]。

正向篩選 (Positive selection) 轉基因植物法

在植物基因轉殖的過程中，篩選轉殖成功之細胞或轉基因植物最常使用的方法是將適當的篩選標示基因 (抗生素抗性基因或殺草劑抗性基因最常見) 連同所欲轉殖的基因同時送入植物細胞內，使未轉殖成功的細胞於選擇性培養基的篩選過程中死亡，以便篩選出轉殖株。除此之外，亦可利用正向篩選 (positive selection) 轉基因植物法以生長代謝相關的基因置換一般使用之抗生素抗性基因或殺草劑抗性基因，藉生化活性的機制依植物生長情形的優劣或生長表型等進行篩選[106] (表二)。目前應用於正向篩選法的基因種類主要有：(1) 可將對細胞不具毒害之特定物質轉變為植物基本生長元素來源 (如碳、氮源) 之基因，例如 β-glucuronidase gene (gusA) 可使轉殖細胞生長於含有 cytokinin glucuronide之培養基[60]、xylose isomerase gene (xylA) 可使轉殖細胞生長於以 D-xylose 為碳源的培養基[43, 44] 及 phosphomannose isomerase gene (manA) 使轉殖細胞可生長於以 D-mannose 為碳源的培養基 [61, 78]。(2) 將對細胞具有毒性之特定物質轉變為無毒性物質之相關基因，其機制類似抗生素抗性或殺草劑抗性基因，如 D-amino acid oxidase gene (dao1) 可將對植物有毒害的 D-amino acid 轉化為對植物無毒害的物質[34]、甜菜鹼醛脫氫瓷 (betaine aldehyde dehydrogenase gene, BADH) 可將對植物有毒性的 betaine aldehyde 轉化為對植物無毒害的 glycine betaine[103]。(3) 植物賀爾蒙相關基因以促進轉殖植物之生長及分化作用，例如 ipt 基因是由 Agrobacterium tumefaciens PO22 選殖而來，為異戊烯基轉移瓷 (isopentenyl transferase) 可催化細胞分裂素 (cytokinin) 的合成，加速轉基因細胞的增生及不定芽的分化 (adventitious shoots)[3]。又如 rol (root locus) 基因含有 A, B, C 三種基因是來自 *A. rhizogenes* NIAES1724，具有提升生長素 (auxin) 靈敏性的功能因而促使毛根 (hairy roots) 的大量增生[96, 108]，因此轉殖成功之植物會表現特殊的外表形態。

除了可利用生長代謝相關基因進行正向篩選之外，亦可使用其他對環境無害或是爭議性較低之基因，例如植物抗病基因，取代一般使用之篩選標示基因。其中自甜椒分離之 ferredoxin-like protein gene (*pflp*) 已被證實可使植物抗軟腐病菌 *Erwinia carotovora* [114]。若於篩選轉殖植物的過程中加入 *E. carotovora* 進行感染，則未轉殖成功之培植體 (explants) 會快速軟化並轉為褐色死亡，而轉殖成功之培植體則仍會維持綠色及正常生長，此方法已於蘭花上施行並獲得證實，且其篩選時間比一般使用之抗生素來得快 [114]。但此類策略較不符合「無篩選標示基因」之宗旨，因為篩選用之基因尚存於植物細胞內。

此外另有一套結合 DNA 序列移除系統 (如：特定位置重組瓷或跳躍子系統) 及正向篩選法之轉殖系統，稱之為 MAT (multi auto transformation) [26]，可將替代的篩選標示基因移除。此法最初成功施行於利用農桿菌中之致癌基因 (oncogenes) 作為篩選標示基因的轉殖載

表二、產生無篩選標示基因轉基因植物之正向篩選法

Table 2. Methods used to generate marker-free transgenic plants by positive selection

Agent and analogues	Genes	Enzymes	Sources	Genome	References
Toxic drugs, metabolite analogues and enzymes used for the conditional-positive selection of transgenic plants					
2-Deoxyglucose	*DOG*^{R}1	2-Deoxyglucose-6-phosphate phosphatase	*Saccharomyces cerevisiae*	Nuclear	68
Betaine aldehyde	*BADH*	Betaine aldehyde dehydrogenase	*Spinacia oleracea*	Nuclear, plastid	103; 15
S-Aminoethyl l-cysteine (AEC)	*DHPS*	Dihydropicolinate synthase	*Escherichia coli*	Nuclear	82;
	ocs	Octopine synthase	*Agrobacterium tumefaciens*		65
4-Methyltryptophan (4-mT)	TDC	Tryptophan decarboxylase	*Catharanthus roseus*	Nuclear	39
Methotrexate	DHFR	Dihydrofolate reductase mouse	*Escherichia coli*	Nuclear	30; 48
			Candida albicans	Nuclear	56
D-amino acid	*dao1*	D-amino acid oxidase	yeast	Nuclear	34
Non-toxic agents and enzymes used for the conditional-positive selection of transgenic plants					
D-Xylose	*xylA*	Xylose isomerase	*Streptomyces rubignosus*, *Thermoanaerobacterium sulfurogenes*	Nuclear	43; 44
D-Mannose	*manA* (*pmi*)	Phosphomannose isomerase	*Escherichia coli*	Nuclear	61; 78
Benzyladenine-*N*-3-glucuronide	*uidA*(*gusA*)	β-Glucuronidase	*Escherichia coli*	Nuclear	60
Enzymes used for the non-conditional-positive selection of transgenic plants					
None	*ipt*	Isopentyl transferases	*Agrobacterium tumefaciens*	Nuclear	31;
	pga 22		*Arabidopsis thaliana*		121
None	*rol*	"Hairy root" phenotype	*Agrobacterium rhizogenes*	Nuclear	27
None	*ESR1*	Transcription factor (enhancer of shoot regeneration 1)	*Arabidopsis thaliana*	Nuclear	4
None	*CKI1*	Histidine kinase (cytokinin-independent 1)	*Arabidopsis thaliana*	Nuclear	121
Erwinia carotovora	*pflp*	ferredoxin-like protein	*Capsicum annuum L.*	Nuclear	114

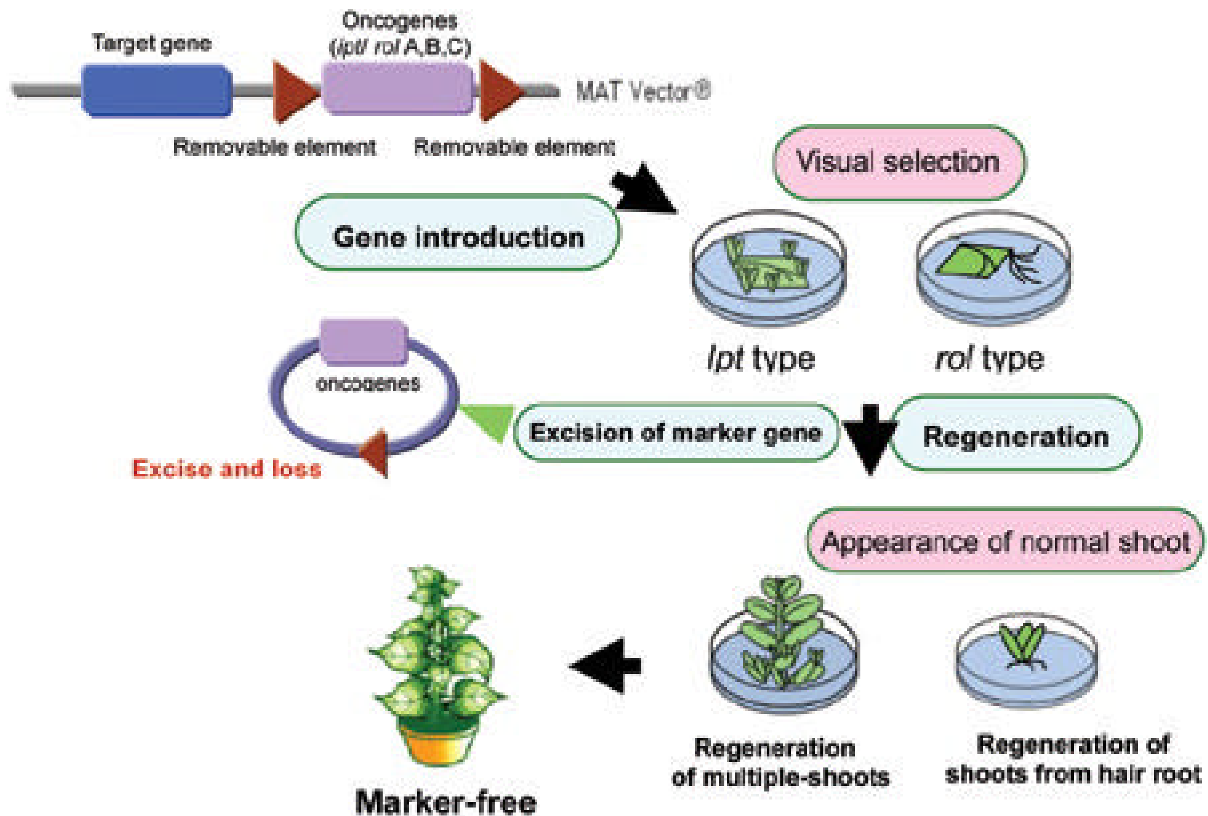
(modified from Ref. 75)

體系統中。因致癌基因有兩種 (即 *ipt* gene 及 *rol* gene)，故可分為 *ipt*- 型載體 (*ipt*-type vector)[25, 28, 33, 101]及 *rol*- 型載體 (rol-type vector)[29]兩大類。由於致癌基因會於農桿菌感染後誘導寄主植物產生腫瘤 (crown galls) 或毛根 (hairy roots) 的現象，因此轉殖成功之植物會產生特殊的外表形態，接著即可透過 MAT 載體上所攜帶之特定位置重組酶或跳躍子系統將致癌基因移除，使轉殖植物恢復正常之生長形態 (圖五)。為了進一步調控移除基因的時機及提高效率，於是更進一步利用由玉米中分離出之谷胱啟肽 S 轉移酶 (glutathione-S-transferase, GST-II-27) 基因的啟動子來控制特定位置重組酶的表現， GST-II-27 啟動子可藉由一種殺草劑解毒劑 (herbicide antidote) "Safener"來誘導，藉以調控特定位置重組酶的表現的時機，此系統稱之為 GST-MAT 載體[32, 100]。此法不需重複地進行轉殖，亦不需經子代分離即可獲得無篩選標示基因的轉基因植物，大大的縮短培育的時間並較適用於木本植物上。

除了透過移除、重組或是替代性篩選標示基因的系統獲得不含抗生素抗性或殺草劑抗性基因之轉基因植物外，亦可直接使用不帶篩選標示基因而具強毒力之農桿菌株 (virulent *A. tumefaciens* strain) 進行轉殖[22]，如此一來就可直接獲得無篩選標示基因之轉基因植物而省去子代分離或是移除篩選標示基因的步驟，但仍需耗費大量資材以聚合酶鏈反應 (polymerase chain reaction, PCR) 篩選每棵自培植體抽出之幼芽，且其獲得無篩選標示基因之效率偏低。

葉綠體轉殖 (Chloroplast transformation)

葉綠體 (chloroplast) 轉殖系統於 1988 年首先於單細胞藻類 (*Chlamydomonas reinhardtii*)[7]，隨後在菸草[102]上証明可穩定地將外源 DNA 轉殖至葉綠體之基因體內，目前也可應用於一些重要經濟作物[14]。相較於一般之細胞核轉殖系統，葉綠體轉殖技術擁有某些優點，包括其基因的遺傳屬於母系遺傳，故所轉入的外來基因較不會經由授粉的過程散播至環境當中，降低基因污染的風險[92]。在綠色葉片的單一細胞中含有約 100 個



圖五、MAT vector 之轉殖原理。MAT vector 以致癌基因作為篩選標示基因，並結合 DNA 序列移除系統 (如:位置專一性重組瓷或跳躍子系統) 於轉殖篩選的過程中移除。

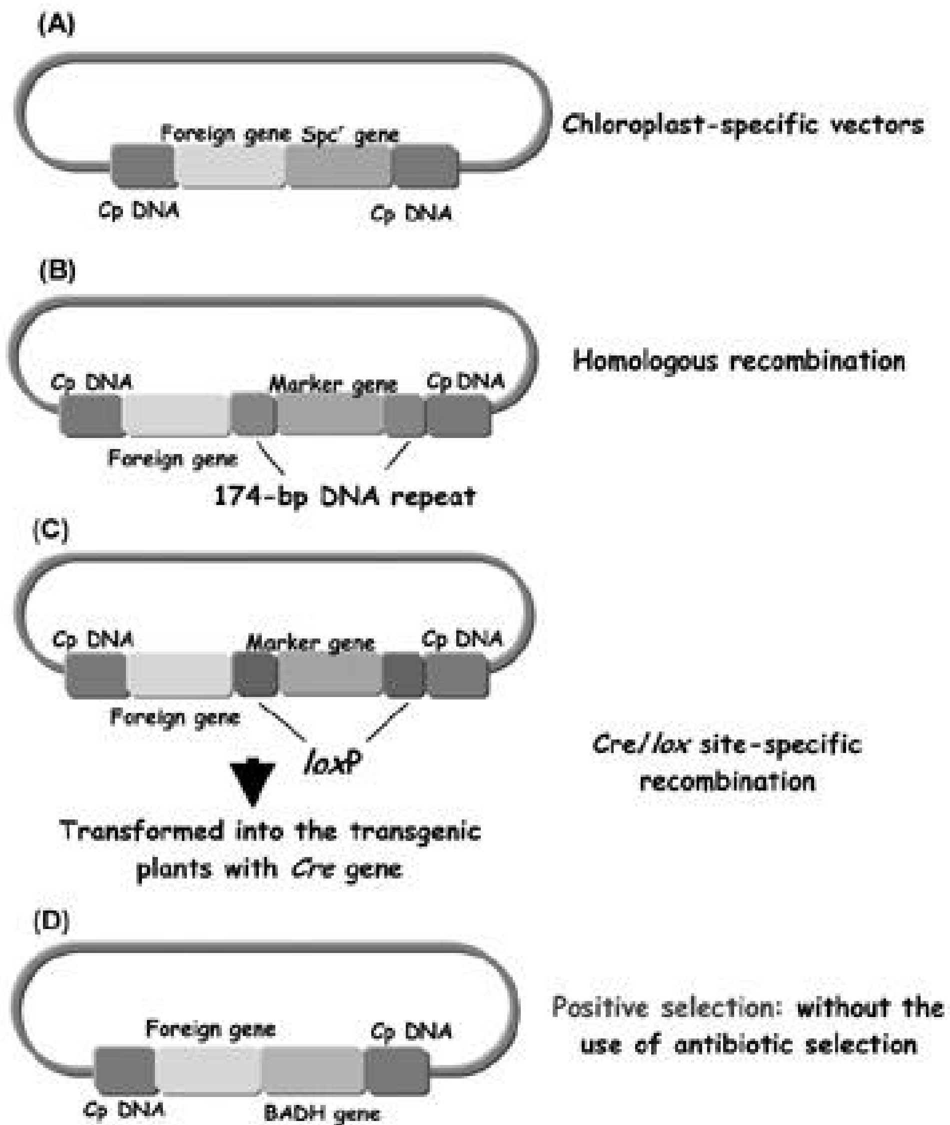
Fig. 5. Principle of the MAT vectors. MAT vectors utilize oncogenes (*ipt* or *rol* genes) as selectable markers and a removal element (site-specific recombination or transposon system) to remove them after transformation (modified from Ref. 27, 28, 29).

的葉綠體，每個葉綠體中又含有約百個高套數的原核基因體[5, 99]，因而能大量地表現外源基因[73, 112]。此外，外來基因在葉綠體中尚未發現產生基因沉寂 (gene silencing) 的現象，因此葉綠體轉殖系統已成為近年新興且受矚目之生物技術。

目前葉綠體轉殖系統除於菸草[102]及阿拉伯芥[94]等模式植物被建立之外，近來在重要經濟作物的葉綠體轉殖技術亦有突破性的進展，包括馬鈴薯[93]、番茄[89]、棉花[66]、大豆[24, 116]、油菜 (oilseed rape and oilseed Brassicacea-*Lesquerella fendleri*)[54, 95]及胡蘿蔔[67]等。雖然葉綠體轉殖系統確有許多優點，但尚有一些瓶頸待突破，目前的技術僅可用於上述少數作物，其他植物往往因缺乏良好的組織培養及再生系統、適當的篩選標示基因或是無法於原質體 (proplastid)、白色體 (leucoplast) 及澱粉體 (amyloplast) 等非綠質體上 (non-green plastid) 表現出轉基因等限制因子，而無法利用葉綠體轉殖系統生產轉基因植物[14]，尤其是世界主要糧食作物如水稻、玉米及小麥等作物仍尚未有成功的案例。因此，葉綠體轉殖系統仍持續地有更多改良之方式正在研究中，以期能提供更廣泛之應用。

以葉綠體轉殖技術將外源基因及篩選標示基因 (一般多為抗生素 spectinomycin) 插入源自葉綠體序列片段之間，構成葉綠體轉殖專用之特殊載體 (圖六A)，經由基因槍法[102]或聚乙烯二醇法 (polyethylene glycol, PEG)[80]送入目標細胞後，少數之載體即有機會進入葉綠體中並透過同源重組的機制將載體上位於葉綠體序列片段間之外來基因嵌入葉綠體基因組中，經由不斷的分化及篩選，細胞內就充滿了攜帶外來基因之葉綠體。由於葉綠體轉殖技術已成為近來植物基因轉殖技術之利器，而朝向發展無篩選標示基因轉基因植物更為當前重要之研究指標，故建立無篩選標示基因轉殖系統亦是葉綠體轉殖技術之重要課題 (表三)。

葉綠體轉殖技術之原理主要是建構於同源重組的機制上，因此亦可應用相同之原理將篩選標示基因移除[17]，例如於葉綠體轉殖專用之特殊載體上的篩選標示基因之兩端各再加入來自葉綠體 174 個鹼基對 (base pair) 之 DNA 序列後進行轉殖 (圖六B)，初期以抗生素篩選出成功轉殖入葉綠體之細胞，經一段時間後再移



圖六、產生無篩選標示基因葉綠體轉基因植物之方法。Cp DNA: 指葉綠體 DNA 序列。
Fig. 6. Methods used to generate marker-free chloroplast transgenic plants. Cp DNA: chloroplast DNA sequence.

表三、產生無篩選標示基因葉綠體轉基因植物之方法
Table 3. Methods used to generate marker-free chloroplast transgenic plants

Methods	Crops	References
Homologous recombination	Tobacco	55
Positive selection (BADH)	Tobacco	15
Cre/lox-mediated recombination	Tobacco	9, 42
Transiently cointegrated selection gene	Tobacco	63

植至無篩選壓力下的環境生長，使篩選標示基因無須被持續性的表現，即可再經同源重組的機制將篩選標示基因和標的基因分離 (圖六B) [55]。此外，葉綠體轉殖系統亦可結合 Cre/*lox* 特定位置重組法或跳躍子系統獲得無篩選標示基因轉殖植物，只需將供辨識之正向重複序列置於欲移除之基因兩端即可 (圖六C)，惟需將 *cre* 基因轉殖至細胞核中，使其於細胞核中表現再進入葉綠體後才能發揮效果[9, 42]。此外，亦可利用正向篩選法將篩選標示基因以來自菠菜之甜菜鹼醛氫瓷 (BADH) 替代，藉其可將具毒性之 betaine aldehyde (BA) 轉換成無毒之 glycine betaine (GB) 進行篩選，同樣可獲得無抗生素抗性基因之轉殖株，且效率較傳統之

spectinomycin 篩選提升 25 倍 (圖六D) [15]。近來還有一套基於同源重組的機制的新轉殖系統 -transiently cointegrated selection gene- 被建立，其策略是將載體上之篩選標示基因置於同源序列的外側，且只有外源基因的兩端具有同源序列，於轉殖過程的短暫時間裡即有機會發生單側之同源重組，而使整個載體嵌入葉綠體基因組中，但此時之構造是不穩定的。由於外源基因的兩端具有同源序列，故可再次進行重組而穩定地嵌入於葉綠體基因組中，並不需經子代分離即可與篩選標示基因分離[63]。

結　論

發展植物基因轉殖技術最終目的在於培育出具有商業或實用價值之轉基因作物，以提供農業應用、提升食品品質及改善人體健康等用途。因此，自轉基因作物中移除在植物內不具任何實質功能之抗生素抗性或殺草劑抗性這類篩選標示基因已成為重要之研究指標。過去數年來已陸續發展出多種不同形式之生產無篩選標示基因轉基因植物的轉殖系統 (表一、二、三)，且持續有更多改良的方式正在研究中。雖然有多種策略可供選擇，但仍需依植物之特性及技術成熟與否採用最適當的方法進行轉殖。此外，適用之作物不再只侷限於實驗模式植物如菸草等，而是擴展至其他許多具有經濟價值的作物如水稻、玉米及大豆等。因此未來釋放至田間的轉基因作物即不再需要抗生素抗性或抗殺草劑抗性之篩選標示基因的存在，可大大降低轉基因作物對環境的衝擊及大眾的疑慮，並更符合市場之需求。

目前在我國除了中研院生農所已開發出一套利用分離自甜椒對軟腐病菌 (*E. carotovora*) 具抗性之基因 (*pflp*) 作為正向篩選產生不具抗生素抗性或殺草劑抗性基因之轉基因植物外[114]，並未有其他可真正地將篩選標示基因移除之轉殖系統被建立。此外，有鑑於多數發表的轉殖方法已被申請專利，因此本實驗室採用其中一種最簡單的轉殖方法-共轉型法；透過無篩選標示基因二位元載體構築，採用雙載體／單菌株及雙 T-DNA/單載體法進行試驗，已發展出一套能有效率地生產無篩選標示基因之轉殖系統。

誌　謝

本實驗室關於"產生無篩選標示基因轉基因植物之二位元載體之構築"承行政院國家科學委員會大學學術追求卓越發展延續計畫 (NSC93-2752-B-005-002-PAE) 經費補助，特示謝忱。

引用文獻 (LITERATURE CITED)

1. Afolabi, A. S., Worland, B. J., Snape, W., and Vain, P. 2004. A large-scale study of rice plants transformed with different T-DNAs provides new insights into locus composition and T-DNA linkage configurations. Theor. Appl. Genet. 109: 815-826.
2. Afolabi, A. S., Worland, B. J., Snape, W., and Vain, P. 2005. Novel pGreen/pSoup dual-binary vector system in multiple T-DNA co-cultivation as a method of producing marker-free (clean gene) transgenic rice (*Oriza sativa* L.) plant. Arf. J. Biotechnol. 4: 531-540.
3. Akiyoshi. D. E., Klee, H., Amasino, R. M., Nester, E. W., and Gordon, M. P. 1984. T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. Proc. Natl. Acad. Sci. U S A. 81: 5994-5998.
4. Banno, H., and Chua, N.-H., 2002. Esr1-a plant gene that can promote plant regeneration and transformation. US Patent Application 20, 020, 157, 140.
5. Bendich, A. J. 1987. Why do chloroplasts and mitochondria contain so many copies of their genome? BioEssays 6: 279-282.
6. Bennett, P. M., Livesey, C. T., Nathwani, D., Reeves, D. S., Saunder, J. R., and Wise, R. 2004. An assessment of the risks associated with the use of antibiotic resistance genes in genetically modified plants: report of the Working Party of the British Society for Antimicrobial Chemotherapy. J. Antimicrob. Chemother. 53: 418-431.
7. Boynton, J. E., Gillham, N. W., Harris, E. H., Hosler, J. P., Johnson, A. M., Jones, A. R., Randolph-Anderrson, B. L., Robertson, D., Klein, T. M., Shark, K.B., and Sanford, J. C. 1988. Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. Science 240: 1534-1538.
8. Chen, S., Li, X., Liu, X., Xu, H., Meng, K., Xiao, G., Wei, X., Wang, F., and Zhu, Z. 2005. Green fluorescent protein as a vital elimination marker to easily screen marker-free transgenic progeny derived from plants co-transformed with a double T-DNA binary vector system. Plant Cell Rep. 23:625-31.
9. Corneille, S., Lutz, K., Svab, Z., and Maliga, P. 2001. Efficient elimination of selectable marker genes from the plastid genome by the CRE-*lox* site-specific recombination system. Plant J. 27: 171-178.
10. Cotsaftis, O., Sallaud, C., Breitler, J. C., Meynard, D., Greco, R., Pereira, A., and Guiderdoni, E. 2002. Transposon-mediated generation of T-DNA and marker free rice plants expressing a Bt endotoxin gene. Mol. Breed. 10: 165-180.
11. Dale, E. C., and Ow, D. W. 1991. Gene transfer with

subsequent removal of the selection gene from the host genome. Proc. Natl. Acad. Sci. USA 23: 10558-10562.
12. Dale, P. J., Clarke, B., and Fontes, E. M. G. 2002. Potential for the environmental impact of transgenic crops. Nat. Biothchnol. 20: 567-574.
13. Daley, M., Knauf, V. C., Summerfelt, K. R., and Turner, J. C. 1998. Co-transformation with one *Agrobacterium tumefaciens* strain containing two binary plasmids as a method for producing marker-free transgenic plants. Plant Cell Rep. 17: 489-496.
14. Daniell, H., Kumar, S., and Dufourmantel, N. 2005. Breakthrough in chloroplast genetic engineering of agronomically important crops. Trends Biotechnol. 23: 238-245.
15. Daniell, H., Muthukumar, B., and Lee, S. B. 2001. Marker free transgenic plants: engineering the chloroplast genome without the use of antibiotic selection. Curr. Genet. 39: 109-116.
16. Davies, G. j., Kilby, J., Riou-Khamlichi, C., and Murray, J. A. H. 1999. Somatic and germinal inheritance of an FLP-mediated deletion in transgenic tobacco. J. Exp. Bot. 50: 1447-1456.
17. Day, A., Kode, V., Madesis, P., and Iamtham, S. 2005. Simple and efficient removal of marker genes from plastids by homologous recombination. Methods Mol. Biol. 286: 255-270.
18. de Block, M., and Debrouwer, D. 1991. Two T-DNA's co-transformed into *Brassica napus* by a double *Agrobacterium tumefaciens* infection are mainly integrated at the same locus. Theor. Appl. Genet. 82: 257-263.
19. de Framond, A. J., Back, E. W., Chilton, W. S., Kayes, L., and Chilton, M. D. 1986. Two unlinked T-DNAs can transform the same tobacco plant cell and segregate in the F1 generation. Mol. Gen. Genet. 202: 125-131.
20. de Neve, M., de Buck, S., Jacobs, A., van Montagu, M., and Depicker, A. 1997. T-DNA integration patterns in co-transformed plant cells suggest that T-DNA repeats originate from co-integration of separate T-DNAs. Plant J. 11: 15-29.
21. Depicker, A., Herman, L., Jacobs, A., Schell, J., and van Montagu, M. 1985. Frequencies of simultaneous transformation with different T-DNAs and their relevance to the *Agrobacterium*/plant cell interaction. Mol. Gen. Genet. 201: 477-484.
22. de Vetten, N., Wolters, A. M., Raemakers, K., van der Meer, I., ter Stege, R., Heeres, E., Heeres, P., and Visser, R. 2003. A transformation method for obtaining marker-free plants of a cross-pollinating and vegetatively propagated crop. Nat. Biotechnol. 21: 439-442.
23. Dröge, M., Pühler, A., and Selbitschka, W. 1998. Horizontal gene transfer as a biosafety issue: A natural phenomenon of public concern. J. Biotechnol. 64: 75-90.
24. Dufourmantel, N., Pelissier, B., Garcon, F., Peltier, G., Ferullo, J. M., and Tissot, G. 2004. Generation of fertile transplastomic soybean. Plant Mol. Biol. 55: 479-489.
25. Ebinuma, H., and Komamine, A. 2001. MAT (multi-auto-transformation) vector system. The oncogenes of *Agrobacterium* as positive markers for regeneration and selection of marker-free transgenic plants. In Vitro Cell. Dev. Biol.-Plant 37: 103-113.
26. Ebinuma, H., Sugita, K., Endo, S., Matsunaga, E., and Yamana, K. 2005. Elimination of marker genes from transgenic plants using MAT vector systems. Methods Mol. Biol. 286: 237-254.
27. Ebinuma, H., Sugita, K., Matsunaga, E., Endo, S., Yamada, K., and Komamine, A. 2001. Systems for the removal of a selection marker and their combination with a positive marker. Plant Cell Rep. 20: 383-392.
28. Ebinuma, H., Sugita, K., Matsunaga, E., and Yamakado, M. 1997a. Selection of marker-free transgenic plants using the isopentenyl transferase gene. Proc. Natl. Acad. Sci. USA 94: 2117-2121.
29. Ebinuma, H., Sugita, K., Matsunaga, E., Yamakado, M., and Komamine, A. 1997b. Principle of MAT vector. Plant Biotechnol. 14: 133-139.
30. Eichholtz, D. A., Rogers, S. G., Horsch, R. B., Klee, H. J., Hayford, M., Hoffman, N. L., Braford, S. B., Fink, C. F., Flick, J., O'Connell, K. M., and Fraley, R. T. 1987. Expression of mouse dihydrofolate reductase gene confers methotrexate resistance in transgenic petunia plants. Somatic Cell Mol. Genet. 13: 67-76.
31. Endo, S., Kasahara, T., Sugita, K., Matsunaga, E., and Ebinuma, H. 2001. The isopentyl transferase gene is effective as a selectable marker gene for plant transformation in tobacco (*Nicotiana tabacum* cv. Petite Havana SR1). Plant Cell Rep. 20: 60-66.
32. Endo, S., Kasahara, T., Sugita, K., and Ebinuma, H. 2002a. A new GST-MAT vector containing both *ipt* and *iaaM/H* genes can produce marker-free transgenic tobacco plants with high frequency. Plant Cell Rep. 20: 923-928.
33. Endo, S., Sugita, K., Sakai, M., Tanaka, H., and Ebinuma, H. 2002b. Single-step transformation for generating marker-free transgenic rice using the *ipt*-type MAT vector system. Plant J. 30: 115-122.
34. Erikson, O., Hertzberg, M., and Näsholm, T. 2004. A conditional marker gene allowing both positive and negative selection on plants. Nat. Biotechnol. 22: 455-458.

35. FAO/WHO. 2000. Safety aspects of genetically modified foods of plant origin. Report of a Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology.
36. Fedoroff, N. 2000. Transposons and genome evolution in plants. Proc. Natl. Acad. Sci. USA 97: 7002-7007.
37. Gidoni, D., Bar, M., Leshem, B., Gilboa, N., Mett, A., and Feiler, J. 2001. Embryonal recombination and germline inheritance of recombined *FRT* loci mediated by constitutively expressed FLP in tobacco. Euphytica 121: 145-156.
38. Gleave, A. P., Mitra, D. S., Mudge, S. R., and Morris, B. A. M. 1999. Selectable marker-free transgenic plants without sexual crossing: transient expression of *cre* recombinase and use of a conditional lethal dominant gene. Plant Mol. Biol. 40: 223-235.
39. Goddijn, O. J. M., van der Duyn Schouten, P. M., Schilperoort, R. A., and Hoge, J. H. C. 1993. A chimeric tryptophan decarboxylase gene as a novel selectable marker in plant cells. Plant Mol. Biol. 22: 907 – 912.
40. Goldsbrough, A. P., Lastrella, C. N., and Yoder, J. I. 1993. Transposition-mediated re-positioning and subsequent elimination of marker genes from transgenic tomatoes. Bio/Technology 11: 1286-1292.
41. Gorbunova, V., and Levy, A. A. 2000. Analysis of extrachromosomal *Ac/Ds* transposable elements. Genetics 155: 349-359.
42. Hajdukiewicz, P. T., Gilbertson, L., and Staub, J. M. 2001. Multiple pathways for Cre/*lox*-mediated recombination in plastids. Plant J. 27: 161-170.
43. Haldrup, A., Petersen, S. G., and Okkels, F. T. 1998a. Positive selection: a plant selection principle based on xylose isomerase, an enzyme used in the food industry. Plant Cell Rep. 18: 76-81.
44. Haldrup, A., Petersen, S. G., and Okkels, F. T. 1998b. The xylose isomerase gene from *Thermoanaerobacterium thermosulfurogenes allows effective* selection of transgenic plant cells using D-xylose as the selection agent. Plant Mol. Biol. 37: 287-296.
45. Hare, P. D., and Chua, N. H. 2002. Excision of selectable marker genes from transgenic plants. Nat. Biotechnol. 20: 575-580.
46. Haring, M. A., Rommens, C., Nijdamp, H. J. J. and Hille, J. 1991. The use of transgenic plants to understand transposition mechanisms and to develop transposon taggign strategies. Plant Mol. Biol. 16: 449-461.
47. Heritage, J. 2004. The fate of transgenes in the human gut. Nat. Biotechnol. 22: 170-172.
48. Herrera-Estrella, L., de Block, M., Messens, E., Hernalsteen, J.-P., van Montagu, M., and Schell, J. 1983. Chimeric genes as dominant selectable markers in plant cells. EMBO J. 2: 987-995
49. Herrera-Estrella, L., Simpson, J., and Martinez-Trujillo, M. 2005. Transgenic plants: an historical perspective. Methods Mol. Biol. 286: 3-32.
50. Hoa, T. T., Bong, B. B., Huq, E., and Hodges, T. K. 2002. Cre/*lox* site-specific recombination controls the excision of a transgene from the rice genome. Theor. Appl. Genet. 104: 518-525.
51. Hoff, T., Schnorr, K. M., and Mundy, J. 2001. A recombinase-mediated transcriptional induction system in transgenic plants. Plant Mol. Biol. 45: 41-49.
52. Hohn, B., Levy, A. A., and Puchta, H. 2001. Elimination of selection markers from transgenic plants. Curr. Opin. Biotechnol. 12: 139-143.
53. Horsch, R. B., Fry, J. E., Hoffmann, H. L., Eichholtz, D., Rogers, S. G., and Fraley, R. T. 1985. A simple and general method for transferring genes into plants. Science 227: 1229-1230.
54. Hou, B. K., Zhou, Y. H., Wan, L. H., Zhang, Z. L., Shen, G. F., Chen, Z. H., and Hu, Z. M. 2003. Chloroplast transformation in oilseed rape. Transgenic Res. 12: 111-114.
55. Iamtham, S., and Day, A. 2000. Removal of antibiotic resistance genes from transgenic tobacco plastids. Nat. Biotechnol. 18: 1172-1176.
56. Irdani, T., Bogani, P., Mengoni, A., Mastromei, G., and Buiatti, M. 1998. Construction of a new vector conferring methotrexate resistance in *Nicotiana tabacum* plants. Plant Mol. Biol. 37: 1079-1084.
57. Jacob, S. S., and Veluthambi, K. 2002. Generation of selection marker-free transgenic plants by cotransformation of a cointegrate vector T-DNA and a binary vector T-DNA in one *Agrobacterium tumefaciens* strain. Plant Sci. 163: 801-806.
58. Jaiwal, P. K., Sahoo, L., Singh, N. D., and Singh, R. P. 2002. Strategies to deal with the concern about marker genes in transgenic plants: Some environment-friendly approaches. Curr. Sci. 83: 128-136.
59. Jin, W. Z., Duan, R. J., Zhang, F., Chen, S. Y., Wu, Y. R., and Wu, P. 2003. Application of *Ac/Ds* transposon system to genetate marker gene free transgenic plants in rice. Sheng Wu Gong Cheng Xue Bao. 19: 668-673. (In Chinese)
60. Joersbo, M., and Okkels, F. T. 1996. A novel principle for selection of transgenic plant cells: positive selection. Plant Cell Rep. 16: 219-221.
61. Joersbo, M., Donaldson, I., Kreiberg, J., Petersen, S. G., Brundstedt, J., and Okkels, F. T. 1998. Analysis of mannose selection used for transformation of sugar beet. Mol. Breed. 4: 111-117.

62. Kilby, N. J., Davies, G. J., and Snaith, M. R. 1995. FLP recombinase in transgenic plants. Nat. Biotechnol. 3: 215-216.
63. Klaus, S. M., Huang, F. C., Gold, T. J., and Koop, H. U. 2004. Generation of marker-free plastid transformants using a transiently cointegrate selection gene. Nat. Biotechnol. 22: 225-229.
64. Komari, T., Hiei, Y., Saito, Y., Murai, N., and Kumashiro, T. 1996. Vectors carrying two separate T-DNAs for co-transformation of higher plant mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. Plant J. 10: 165-174.
65. Koziel, M. G., Adams, T. L., Hazlet, M. A., Damm, D., Miller, J., Dahlbeck, D., Jayne, S., and Staskawics, B. J. 1984. A cauliflower mosaic virus promoter directs expression of kanamycin resistance in morphogenic transformed plant cells. J. Mol. Appl. Genet. 2: 549 – 562
66. Kumar, S., Dhingra, A., and Daniell, H. 2004a. Stable transformation of the cotton plastid genome and maternal inheritance of transgenes. Plant Mol. Biol. 56: 203-216.
67. Kumar, S., Dhingra, A., and Daniell, H. 2004b. Plastid-expressed *betaine aldehyde dehydrogenase* gene in carrot cultured cells, roots, and leaves confers enhanced salt tolerance. Plant Physiol. 136: 2843-2854.
68. Kunze, I., Ebneth, M., Heim, U., Geiger, M., Sonnewald, U., and Herbers, K. 2001. 2-Deoxyglucose resistance: a novel selection marker for plant transformation. Mol. Breed. 7: 221-227.
69. Lu, H.-J., Zhou, C. R., Gong, Z.-X., and Upadhyaya, N. M. 2001. Generation of selectable marker-free transgenic rice using double right border (DRB) binary vectors. Aust. J. Plant Physiol. 28: 241-248.
70. Luo, H., Lyzink, L. A., Gidoni, D., and Hodges, T. K. 2000. FLP-mediated recombination for use in hybrid plant production. Plant J. 23: 423-430.
71. Lyznik, L. A., Rao, K.V., and Hodges, T. K. 1996. FLP-mediated recombination of FRT sites in the maize genome. Nucl. Acids Res. 24: 3784-3789.
72. Matthew, P. R., Wang, M.-B., Waterhouse, P. M., Thornton, S., Fieg, S. J., Gubler, F., and Jacobsen, J. V. 2001. Marker gene elimination from transgenic barley, using co-transformation with adjacent "twin T-DNAs" on a standard *Agrobacterium* transformatiom vector. Mol. Breed. 7: 195-202.
73. McBride, K. E., Svab, Z., Schaaf, D. J., Hogan, P. S., Stalker, D. M., and Maliga, P. 1995. Amplification of a chimeric *Bacillus* gene in chloroplasts leads to an extraordinary level of an insecticidal protein in tobacco. Bio/Technology 13: 362-365.
74. McKnight, T. D., Lillis, M. T., and Simpson, R. B. 1987. Segregation of genes transferred to one plant cell from two separate *Agrobacterium tumefaciens* strains. Plant Mol. Biol. 8: 439-445.
75. Miki, B., and McHugh, S. 2004. Selection marker genes in transgenic plants: applications, alternatives and biosafety. J. Biotechnol. 170: 193-232.
76. Miller, M., Tagliani, L., Wang, N., Berka, B., and Bidney, D. 2002. High efficiency transgene segregation in co-transformed maize plants using an *Agrobacterium tumefaciens* 2 T-DNA binary system. Transgenic Res. 11: 381-396.
77. Neilsen, k. M., Bones, A. M., Smalla, K., and van Elsas, J. D. 1998. Horizontal gene transfer from transgenic plants to terrestrial bacteria—a rare event? FEMS Microbiol. Rev. 22: 79-103.
78. Negrotto, D., Jolley, M., Beer, S., Wenck, A. R., and Hansen, G. 2000. The use of phosphomannose-isomerase as a selectable marker to recover transgenic maize plants (*Zea mays* L.) via Agrobacterium transformation. Plant Cell Rep. 19: 798-803.
79. Netherwood, T., Martín-Orúe, S. M., O'Donnell, A. G., Gockling, S., Graham, J., Mathers, J. C., and Gilbert, H. J. 2004. Assessing the survival of transgenic plant DNA in the human gastrointestinal tract. Nat. Biotechnol. 22: 204-209.
80. O'Neill, C., Horvath, G. V., Horvath, E., Dix, P. J., and Medgyesy, P. 1993. Chloroplast transformation in plants: polyethylene glycol (PEG) treatment of protoplasts is an alternative to biolistic delivery systems. Plant J. 3: 729-738.
81. Onouchi, H., Nishihama, R., Kudo, M., Machida, Y., and Machida, C. 1995. Visualization of site-specific recombination catalyzed by a recombinase from *Zygosaccharomyces rouxii* in Arabidopsis thaliana. Mol. Gen. Genet. 247: 653-660.
82. Perl, A., Galili, S., Shaul, O., Ben-Tzvi, I., and Galili, G. 1993. Bacterial dihydrodipicolinate synthase and desensitized aspartic kinase: two novel selectable markers for plant transformation. Bio/Technology 11: 715-718.
83. Permingeat, H. R., Alvarez, M. L., Cervigni, G. D., Ravizzini, R. A., and Vallejos, R. H. 2003. Stable wheat transformation obtained without selectable markers. Plant Mol. Biol. 52: 415-419.
84. Puchta, H. 2003a. Marker-free transgenic plants. Plant Cell Tiss. Org. 74: 123-134.
85. Puchta, H. 2003b. Towards the ideal GMP: Homologous recombination and marker gene excision. J. Plant Physiol. 160: 743-754.
86. Puchta, H., Swoboda, P., Gal, S., Blot, M., and Hohn,

B. 1995. Somatic intrachromosomal homologous recombination events in populations of plant siblings. Plant Mol. Biol. 28: 281-292.

87. Rommens, C. M. 2004. All -native DNA transformation: a new approach to plant genetic engineering. Trends Plant Sci. 9: 457-464.
88. Rommens, C. M., Humara, J. M., Ye, J., Yan, H., Richael, C., Zhang, L., Perry, R., and Swords, K. 2004. Crop improvement through modification of the plant's own genome. Plant Physiol. 135: 421-431.
89. Ruf, S., Hermann, M., Berger, I. J., Carrer, H., and Bock, R. 2001. Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. Nat. Biotechnol. 19: 870-875.
90. Russell, S. H., Hoopes, J. L., and Odell, J. T. 1992. Directed excision of a transgene from the plant genome. Mol. Gen. Genet. 234: 49-59.
91. Schaart, J. G., Krens, F. A., Pelgrom, K. T. B., Mendes, O., and Rouwendal, G. J. A. 2004. Effective production of marker-free transgenic strawberry plants using inducible site-specific recombination and a bifunctional selectable marker gene. Plant Biotechnol. J. 2: 233-240.
92. Scott, S. E., and Wilkinson, M. J. 1999. Low probability of chloroplast movement from oilseed rape (*Brassica napus*) into wild *Brassica rapa*. Nat. Biotechnol. 17: 390-392.
93. Sidorov, V. A., Kasten, D., Pang, S. Z., Hajdukiewicz, P. T. J., Staub, J. M., and Nehra, N. S. 1999. Stable chloroplast transformation in potato: use of green fluorescent protein as a plastid marker. Plant J. 19: 209-216.
94. Sikdar, S. R., Serino, G., Chaudhuri, S., and Maliga, P. 1998. Plastid transformation in *Arabidopsis thaliana*. Plant Cell Rep. 18: 20-24.
95. Skarjinskaia, M., Svab, Z., and Maliga, P. 2003. Plastid transformation in *Lesquerella fendleri*, an oilseed Brassicacea. Transgenic Res. 12: 115-122.
96. Slightom, J. L., Durand-Tardif, M., Jouanin, L., and Tepfer, D. 1986. Nucleotide sequence analysis of TL-DNA of *Agrobacterium rhizogenes* agropine type plasmid. Identification of open reading frames. J. Biol. Chem. 261: 108-121.
97. Sreekala, C., Wu, L., Gu, K., Wang, D., Tian, D., and Yin, Z. 2005. Excision of a selectable marker in transgenic rice (*Oryza sativa* L.) using a chemically regulated Cre/*loxP* system. Plant Cell Rep. 24: 86-94.
98. Srivastava, V., and Ow, D. W. 2004. Marker-free site-specific gene integration in plants. Trends Biotechnol. 22: 627-629.
99. Sugiura, M., Hirose, T., and Sugita, M. 1998. Evolution and mechanism of translation in chloroplasts. Annu. Rev. Genet. 32: 437-459.
100. Sugita, K., Kasahara, T., Matsunaga, E., and Ebinuma, H. 2000. A transformation vector for the production of marker-free transgenic plants containing a single copy transgene at high frequency. Plant J. 22: 461-469.
101. Sugita, K., Matsunaga, E., and Ebinuma, H. 1999. Effective selection system for generating marker-free transgenic plants independent of sexual crossing. Plant Cell Rep. 18: 941-947.
102. Svab, Z., Hajdukiewicz, P., and Maliga, P. 1990. Stable transformation of plastids in higher plants. Proc. Natl. Acad. Sci. USA 87: 8526-8530.
103. Ursin, V. M. 1996. Aldehyde dehydrogenase selectable markers for plant transformation. WO/12029.
104. Vain, P., Afolabi, A., Worland, B., and Snape, J. W. 2003. Transgene behavior in populations of rice plants transformed using a new dual binary vector system: pGreen/pSoup. Theor. Appl. Genet. 107: 210-217.
105. Verhoog, H. 2003. Naturalness and the genetic modification of animals. Trends Biotechnol. 21: 294-297.
106. Wenck, A., and Hansen, G. 2005. Positive selection. Methods Mol. Biol. 286: 227-236.
107. Wenck, A., Czako, M., Kanevski, I., and Marton, L. 1997. Frequent collinear long transfer of DNA inclusive of the whole binary vector during *Agrobacterium*-mediated transformation. Plant Mol. Biol. 34: 913-922.
108. White, F. F., Taylor, B. H., Huffman, G. A., Gordon, M. P., and Nester, E. W. 1985. Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of *Agrobacterium* rhizogenes. J. Bacteriol. 164: 33-44.
109. Wolfenbarger, L. L., and Phifer, P. R. 2000. The ecological risks and benefits of genetically engineered plants. Science 290: 2088-2093.
110. Xing, A., Zhang, Z., Sato, S., Staswick, P., and Clement, T. 2000. The use of two T-DNA binary system to derive marker-free transgenic soybeans. *In Vitro* Cell Dev. Biol.-Plant 36: 456-463.
111. Xue, G. P., Patel, M., Johnson, J. S., Smyth, D. J., and Vickers, C. E. 2003. Selectable marker-free transgenic barley producing a high level of cellulase (1,4-beta-glucanase) in developing grains. Plant Cell Rep. 21: 1088-1094.
112. Ye, G. N., Hajdukiewicz, P. T., Broyles, D., Rodriguez, G., Xu, C.W., Nehra, N., and Staub, J.M. 2001. Plastid-expressed 5-enolpyrulylshikimate-3-phosphate synthase genes provide high level glyphosate tolerance in tobacco. Plant J. 25: 261-270.
113. Yoder, J. I., and Goldsbrough, A. P. 1994. Transformation systems for generating marker-free

transgenic plants. Bio/Technology 12: 263-267.

114. You, S. J., Liau, C. H., Huang, H. E., Feng, T. Y., Prasad, V., Hsiao, H. H., Lu, J. C., and Chan, M. T. 2003. Sweet pepper ferredoxin-like protein (*pflp*) gene as a novel selection marker for orchid transformation. Planta 217: 60-65.

115. Zhang, W., Subbarao, S., Addae, P., Shen, A., Armstrong, C., Peschke, V., and Gilbertson, L. 2003. Cre/*lox*-mediated marker gene excision in transgenic maize (*Zea mays* L.) plants. Theor. Appl. Genet. 107: 1157-1168.

116. Zhang, X. H., Portis, A. R. Jr., and Wildholm, J. M. 2001. Plastid transformation of soybean suspension cultures. J. Plant Biotechnol. 3: 39-44.

117. Zhou, H. Y., Chen, S. B., Li, X. G., Xiao, G. F., Wei, X. L., and Zhu, Z. 2003. Generation marker-free transgenic tobacco plants by *Agrobacterium*-mediated transformation with double T-DNA binary vector. Acta Botanica Sinica 45: 1103-1108.

118. Zubko, E., Scutt, C., and Meyer, P. 2000. Intrachromosomal recombination between attP regions as a tool to remove selectable marker genes from tobacco transgenes. Nat. Biotechnol. 18: 442-445.

119. Zuo, J., Niu, Q. W., and Chua, N. H. 2000. An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. Plant J. 24: 265-273.

120. Zuo, J., Niu, Q. W., Moller, S. G., and Chua, N. H. 2001. Chemical-regulated, site-specific DNA excision in transgenic plants. Nat. Biotechnol. 19: 157-161.

121. Zuo, J., Niu, Q. W., Ikeda, Y., and Chua, N. H. 2002. Marker-free transformation: increasing transformation frequency by the use of regeneration-promoting genes. Curr. Opin. Biotechnol. 13: 173-180.

Ms. Ching-Yi Lin

Ms. Lin received her B. S. degree in plant pathology from National Chung Hsing University (NCHU), Taichung, Taiwan in 2002. She completed a M.S. degree with Dr. Fuh-Jyh Jan in plant pathology from NCHU in 2004. Her M.S. thesis mainly involved in identification and characterization of Carnation mottle virus (CarMV) isolated from carnation and calla lily in Taiwan. She is currently a Ph. D. student under the direction of Dr. Jan working on the development of marker-free transgenic plants with board resistance to geminiviruses via gene silencing.

Dr. Fuh-Jyh Jan

Dr. Jan is currently an assistant professor at the Department of Plant Pathology, National Chung Hsing University (NCHU), Taichung, Taiwan. He received both his B.S. and M.S. degrees in plant pathology from NCHU in 1986 and 1988. In 1998, he earned a Ph.D. degree in molecular plant pathology from Cornell University, USA. His major expertise is in the molecular biology of plant viruses with special emphasis on tospoviruses and potyviruses. He has also involved in identification and characterization of plant viruses infecting ornamental plants including Phalaenopsis orchid, calla lily, carnation and lisianthus in Taiwan. He has developed a transgenic approach for generating multiple resistance by a chimeric construct that can trigger post-transcriptional gene silencing against different viruses. Currently, he is using this approach for the control of major viruses affecting cucurbitaceous and solanaceous crops.

ABSTRACT

Lin, C,-Y.[1], and Jan, F.- J.[1,2]. 2005. Current development of the strategies for generating marker-free transgenic plants. Plant Pathol. Bull.14:159-176. ([1] Department of Plant Pathology, National Chung Hsing University, Taichung, Taiwan; [2] Corresponding author, E-mail: fjjan@nchu.edu.tw, Fax: +886-4-22854145）

Selectable marker genes are required for the efficient transformation of transgenic plants. In most cases, selection is based on antibiotic or herbicide resistance. The persistence of selectable marker genes in transgenic plants has caused public concerns of environmental safety and human health issues. Thus it is desirable to remove the marker gene prior to the release of transgenic plants. Several strategies including co-transformation, site-specific recombination, transposition, homologous recombination and positive selection have been developed to generate marker-free transgenic plants. The implementation of these strategies in crops will help to expedite widespread public acceptance of transgenic plants. Here, we summarize and discuss these transformation systems and the merits, shortcomings and their future possible development of different approaches.

Key words: selection marker genes, marker-free, transgenic plant, chloroplast transformation, *Agrobacterium*-mediated transformation