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Regulated Expression of Yeast FLP Recombinase in Plant Cells

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BARD Final Scientific Report - "Regulated expression of yeast FLP recombinase in plant cells"

Thomas K Hodges and Hong Luo (USA)

David Gidoni, Anahit Mett, Julia Feiler and Ron Vunch (Israel).

Abstract

Research activities in both our laboratories were directed toward development of control of the FLP/*frt* recombination system for plants. As described in the text of the research proposal, the US lab has been engaged in developing regulatory strategies such as tissue-specific promoters and the steroid-inducible activation of the FLP enzyme while the main research activities in Israel have been directed toward the development and testing of a copper-regulated expression of *flp* recombinase in tobacco (this is an example of a promoter activation by metal ions). The Israeli lab has additionally completed experiments of previous studies regarding factors affecting the efficiency of recombinase activity using both a gain-of-function assay (excisional-activation of a *gusA* marker) and loss of function assay (excision of a *rolC* marker) in tobacco.

Site-specific recombinase systems, in particular the FLP/*frt* and R/RS systems of yeast and the Cre/*lox* system of bacteriophage P1, have become an essential component of targeted genetic transformation procedures both in animal and plant organisms. To provide more flexibility in transgene excisions by the recombinase systems as well as gene targeting, and to widen possible applications, the development of controlled or regulated recombination systems is highly desirable and was therefore the subject of this research proposal. There are a few possible mechanisms to regulate expression of a recombinase system. They include: 1) control of the recombination system by having the target sites (e.g. *frt*) in one plant and the *flp* recombinase gene in another, and bringing the two together by cross fertilization. 2) regulation of promoter activities by external stimuli such as temperature, chemicals, metal ions, etc. 3) regulation of promoter activities by internal signals, i.e. cell- or tissue-specific, or developmental regulation. 4) regulation of enzyme activity by providing cofactors essential for biochemical reactions to take place such as steroid molecules in conjunction with a steroid ligand-binding protein (domains).

During the course of this research our major emphasis have been focused toward studying the feasibility of hybrid seed production in *Arabidopsis*, using FLP/*frt*. Male-sterility was induced using the antisense of a pollen- and tapetum-specific gene, *bcpl1*, isolated from *Arabidopsis*. The sterility inducing gene was flanked by *frt* sites. Upon cross pollination of flowers of male-sterile plants with pollen from FLP-containing plants, viable seeds were produced, and the progeny hybrid plants developed normally. The major achievement from this work is the first demonstration of using a site-specific recombinase to restore fertility in male-sterile plants (see attached paper, Luo et al., Plant J 2000; 23:423-430). The implication from this finding is that site-specific recombination systems can be applied in crop plants as a useful alternative method for hybrid seed production.

Achievements

We have studied the feasibility of using a site-specific recombinase, FLP, in *Arabidopsis* plants, for making plant hybrids. This work shows for the first time efficient and no deleterious effects of the FLP/*frt* system in *Arabidopsis* plants. Using this system, we have successfully eliminated in transgenic male-sterile plants the male-sterility causing elements integrated into the host genome, by crossing with pollen from a plant expressing the FLP recombinase, yielding hybrid seeds (see attached paper, Luo et al., Plant J 2000; 23:423-430). This work has indicated that male-sterility can be induced by any pollen- or tapetum cell-specific gene whose malfunction causes male-sterility. Such a male-sterility causing gene would ultimately be excised from the host genome, restoring fertility. Thus, we have demonstrated that the recombination system offers very high flexibility in designing a sterility-fertility restoration system for hybrid seed production.

Hybrids of most crop plants yield 10-30% more than pure inbred lines. Therefore, development of methods for producing hybrid plants are highly desirable. When available, genic and cytoplasmic male sterility (CMS) have been used in hybrid seed production. In many crops, e.g. corn, where neither system is available, therefore detasseling (removal of the male part of line A) and then pollination by line B for hybrid production is used, however detasseling is costly. Neither detasseling nor cytoplasmic male-sterility systems are available for many economically important crops, such as wheat, soybean, canola, or barley, which are still bred and grown as inbred varieties.

Based on the results of our work, we believe that the site-specific recombination systems can be successfully applied in crop plants as a useful alternative for hybrid seed production (medium term, 5 years).

Other achievements:

a) We have demonstrated for the first time activity of the Cre/*lox* and FLP/*frt* site-specific recombination systems in rice (appendix A). The application of site-specific recombination system in producing hybrid rice is currently underway.

We believe this approach will provide a useful method for hybrid seed production in rice.

- b) We have obtained preliminary results indicating copper-regulated FLP activity in tobacco cells (appendix B). The results are encouraging, however, further experiments are yet required to establish this system in whole plants.
- c) We have demonstrated FLP-mediated activation of *gusA* in embryo (in the *flp* x *frt* F1 hybrids, prior to seed maturation) and germinal inheritance of the recombined *frt* locus to F2 progeny in tobacco (appendix C). While two constitutive-FLP expressing lines were crossed with four *frt-gusA* target lines, efficient recombination was detected only in two crosses (made between one FLP and two *frt-gusA* lines). These findings are in line with the importance of pre-screening for early recombinogenic *flp* x *frt* crosses as an indication for efficient recombination in both somatic and germinal developmental stages.
- d) We have made preliminary attempts to examine FLP-mediated restoration of fertility in *rolC* transgenic tobacco (Appendix D). These experiments include crosses of one FLP expressing line with two *frt* – *rolC* – *frt* male-sterile lines. The partial restoration found in these two crosses is in line with inefficient recombination in both crosses that were examined. According to the results in appendix C (described bellow) however, testing of more *flp* x *frt* crosses is required to raise the probability for finding of a recombination-efficient cross.

Description of the cooperation

Collaborative efforts between the IS and US laboratories were integral part of this project. An FLP expressing binary construct and an *frt-gusA* target binary construct were made in the Israeli laboratory and then sent to the US laboratory. These constructs were used in the studies of the activity and restoration of fertility by the FLP/*frt* system in *Arabidopsis* plants. In 1998, David Gidoni visited the Hodges laboratory (from June 7 to June 9). We exchanged information regarding progress and how we would collaborate during the rest of the project. Many of our planning and coordination has been done through correspondence with most contacts being by email. We had a good relationship and respect each others contributions to this

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)	1			1
Submitted, in review, in preparation			2	2
Invited review papers				
Book chapters				
Books				
Master theses				
Ph.D. theses				
Abstracts				1
Not refereed (proceedings, reports, etc.)				

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)				
Licensed				

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Visits/Meetings	1	1		2
Sabbaticals				
Postdoctorates				

- **Cooperation**, briefly explain whether synergistic, complementary or supportive.

Cooperation was characterized by exchange of plasmid constructions and by fruitful exchange of ideas, knowledge and suggestions between the two laboratories. Prof. Tom Hodges visited Bet Dagan in 1996 and David Gidoni visited Purdue in 1998.

List of publications

Refereed:

1. Luo H, Lyznik LA, Gidoni D and Hodges TK (2000) FLP-mediated recombination for use in hybrid plant production. *Plant J* 23:423-430.

Submitted:

1. Gidoni D, Bar M, Leshem B, Gilboa N, Mett A and Feiler J (Sumbitted) Embryonal recombination and germline inheritance of recombined *frit* loci mediated by constitutively expressed FLP in tobacco
2. Gidoni D, Bar M and Gilboa N (Submitted) FLP/*frit*-mediated reversion of normal phenotypes and development of clonal sectors in rolC transgenic tobacco

Abstracts:

1. Gidoni D, Bar M and Gilboa N (2000) FLP/*frit*-mediated reversion of normal phenotypes and development of clonal sectors in rolC transgenic tobacco (abstract) The 6th International Congress of Plant Molecular Biology, Quebec, Canada.
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project. Our collaborative work was published in a joint paper (Luo et al., Plant J 2000; 23:423-430).

Appedices

Appendix A: Cre- and FLP-mediated site-specific recombination activities in rice.

Both FLP/*frt* and Cre/*lox* constructs have been tested. The basic design was to use *frt* or *lox* sites to flank a blocking piece of DNA which separated a promoter, either maize ubiquitin or CaMV35s, from a *gusA*. T0 plants, obtained from *Agrobacterium*-mediated transformation containing these *frt* or *lox* constructs were crossed to T0 plants containing either *flp* or *Cre* genes, respectively. Since T0 plants were used in these experiments and each parent was hemizygous for either the target sites or the recombinase, only 25% of the progeny would be expected to have both target sites and recombinase genes. Seeds were obtained from these crosses and planted for evaluation. As expected, approximately 25% of the plants did express GUS. Southern blot analyses indicated that FLP did catalyze excision of the blocking DNA, but not as completely as observed in parallel *Arabidopsis* experiments. Southern blots of the Cre/*lox* hybrid plants indicated Cre was very effective in excising all of the blocking DNA. The reason for FLP not working as effectively in rice as in *Arabidopsis* may be due to differences in the two species; however, the constructs containing the *flp* gene for the *Arabidopsis* experiments and the rice experiments were different, therefore further comparative experiments are still required.

Appendix B: Copper-regulated expression of FLP recombinase in tobacco cells

In order to develop copper-inducible FLP recombinase in plant cells, we have made the following DNA constructs:

(a) **p9eJACE:** 4e-35sP Ω -*ace1* - 35st, a binary vector containing *ace1* (coding region of yeast metallothionein gene activator protein) linked to: 1) CaMV 35s promoter (35sP) [that contains four repetitive transcriptional enhancer sequences (4e) and omega translational enhancer (Ω)], and 2) a 35s 3' end terminator (35st).

pUC119.ACE1.6 (kindly provided by Prof. P. Reynolds and V. Mett, New Zealand) was digested with *Hind* III, then dephosphorylated and ligated to a *Hind* III-*Bam* HI-*Hind* III adapter to yield pUACEB. A 0.65 kb *Bam* HI fragment containing *ace 1* coding region was isolated from pUACEB and ligated downstream to the 4 x enhancer-35sP Ω region of a *Bam* HI linearized binary vector pPCV91 (kindly provided by Prof. C.Koncz, Germany). The resulting (11.6 kb) plasmid was termed p9eJACE.

(b) pU4M90FL: 4xMREs - min35sP Ω -FLP, a plasmid containing FLP coding region downstream from *MREs* (binding site of *ace1* on the yeast metallothionein gene promoter) and minimal (-90) (min35sP Ω) upstream control sequences.

pUC119/4MT3 (kindly provided by Prof. P. Reynolds and V. Mett, New Zealand) was digested with *Sac* I and *Sma* I (positioned immediately downstream to the four *MREs*) and ligated to a 1.45 kb *Sac* I - *Eco* RV (partial digest) fragment from pJFLO containing a 35SP Ω -FLP (Luo et al., 2000). The resulting plasmid was termed pU4M90FL.

(c) pU4M90FG: 4xMREs - min35SP Ω -f rt -gusA, a control plasmid construct containing *gusA* linked to [4x*MREs*-min35sP Ω -f rt] upstream sequences.

A 1.9 kb *Sal* I - *Sac* I [f rt - *gusA*] fragment from pJFG (constructed within the framework of this BARD grant, contains *gusA* linked to 35sP Ω -f rt upstream sequences) was used to replace *FLP* coding region in pU4M90FL. The resulting plasmid construct was termed pU4M90FG.

In the next step, efforts were made to introduce the 4xMREs-min35sP Ω -FLP region (from pU4M90FL) and the 4xMREs-min35sP Ω -f rt -gusA (from pU4M90FG) upstream to a *ocs* gene transcriptional terminator signal (*ocst*) inside the binary vector p9eJACE. The expected binary fusion constructs were termed p4M90FL/JACE and pU4M90FG/JACE, respectively.

Construction of the putative p4M90FL/JACE binary plasmid is, due to technical difficulties, still in progress.

Construction of the binary vector p4M90FG/JACE (14.3 Kb) has been completed.

A schematic display of p4M90FG/JACE:

[4e-35sP Ω -*ace1*-35st] [4xMREs-min35sP Ω -f rt -gusA-ocst]

This plasmid was subjected to transfection experiments into plant cells, as described below.

Since construction of p4M90FL/JACE binary vector was encountered with difficulties (related to instability of the construct in *E. Coli*), we introduced a *nos* terminator signal (*nost*, isolated from pJFLO) downstream to *FLP* in p4M90FL, giving rise to pCu5 (5.0 Kb). As a control plasmid, a *nost* fragment was fused downstream to p4M90FG, giving rise to pCu7 (5.5 Kb).

In the next series of experiments, preliminary studies of copper-regulated expression of FLP in plant cells was conducted by introduction of plasmids, using DNA bombardment, into leaf-disks derived from a FFG(601) transformed tobacco line (Bar et al., 1996). This plant line contains a blocking *f rt -neo-f rt* region which has been positioned between 35sP Ω and *gusA* coding sequences in pFFG (Bar et al., 1996). GUS activity was used to monitor FLP-mediated conversion of the silent to an active *gusA*, resulting from excision of the blocking *f rt -neo-f rt* fragment in FFG(601) (Table 1).

Treatments A, D and E in Table 1 represent negative controls, indicating background GUS activities which may be attributed either to bacterial (or other) contamination

(A, D) or residual GUS expression (“leakiness”) derived from the minimal(-90) 35s promoter upstream to *gusA* in pCu7 (E). Treatment B represents constitutive GUS expression from the 35s promoter in pJFG, while the GUS activity in treatment C is expectedly, derived from FLP-mediated excisional recombination of the genomic FFG locus in these plants (Bar et al., 1996).

Copper-mediated binding of ACE1 protein to *MRE* sequences in pCu5 (treatment H) is expected to activate transcription of the downstream linked *FLP* gene, while the binding of Cu^{++} -ACE1 to *MRE* sequences in the control p4M90FG/JACE and pCu7 plasmids (treatments F and G, respectively) is expected to activate transcription of *gusA*. The results of treatments F, G and H shown in experiment 3 (Table 1) indicate background GUS activity. This suggests the presence of copper ions in the cells in spite of the use of copper-omitted media during plant growth. In experiment 2, however, transfer of leaves to copper-containing media (time points 24 and 48 h) was accompanied with moderate rise in GUS activity. These results are consistent with the possibility of copper-mediated activation of both FLP (treatment H, plasmid pCu5) and GUS expression (treatments F and G, plasmids p4m90FG and pCu7, respectively).

The preliminary results described here indicate the feasibility of copper induction of FLP recombinase in plant cells. However, Further optimization studies for copper-regulated FLP in a transient expression system, as well as different alternative strategies have yet to be tested in order to gain stable transformation and facilitate the evaluation of this system in whole plants.

Table 1. Transient GUS expression in copper-induced FLP recombinase in tobacco.

Treatment	Plasmid	Experiment	Number of Gus ⁺ spots per leaf ¹		
			0 h	24 h	48 h
A	pUC (2.7 kb)	1	2	ND	0
		2	0	2	0
		3	0	2	1
B	pJFG (11.2 kb)	1	26	ND	21
		2	31	20	20
		3	96	52	20
C	pJFLO (10.7 kb)	1	11	ND	10
		2	15	12	11
		3	20	30	20
D	pJACE (11.6 kb)	1	1	ND	1
		2	ND	ND	ND
		3	ND	ND	ND
E	pCu7 (5.5 kb)	1	4	ND	4
		2	ND	ND	ND
		3	ND	ND	ND
F	p4M90FG/JACE (14.3 kb)	1	ND	ND	ND
		2	0	40	10
		3	28	21	97
G	pJACE (11.6 kb) + pCu7 (5.5 kb)	1	ND	ND	ND
		2	5	10	19
		3	5	18	2
H	pJACE (11.6 kb) + pCu 5 (5 kb)	1	ND	ND	ND
		2	6	21	10
		3	20	20	2

¹Transgenic FFG(601) tobacco plantlets (derived from cuttings) were grown in sterile conditions in a growth chamber (25°C, 16/8 h light/dark cycles) on MS medium lacking Cu⁺⁺. In each experiment three leaf disks were pre-incubated for 2 h on MS medium containing 0.25M sorbitol in the absence of copper. Each leaf was then bombarded (pressure: 650 psi., distance: 7 cm., according to Bio-Rad, California) with tungsten particles coated with 0.2 µg of total plasmid DNA and transferred to copper-containing media (100 µM CuSO₄, Mett et al., 1993) for 0, 24 and 48 h. GUS activity was detected by histochemical staining and was measured for each experiment as the average number of GUS-positive spots per leaf disk. (ND, not done).

Appendix C: FLP-mediated embryonal recombination and germline transfer of the recombined *frt* loci in tobacco

Better understanding of principles affecting FLP recombination efficiency through somatic and germinal plant stages will be useful in applications where efficient recombination activities would be required.

FLP-mediated activation of *gusA* in early and late stages during embryo germination and inheritance of the recombined *35sPQ-frt-gusA* loci to F₂ generation were studied using constitutively expressed FLP in both pJFL (a non-modified FLP, Bar et al., 1996) and a modified FLP in pJFLO (Luo et al., 2000). The FLP coding sequence in pJFLO was derived from pOG44 (O'Gorman et al., 1991) and the sequence immediately upstream to the ATG initiation codon was modified from CACC to AACA, consistent with the consensus sequence for plants (Lutcke et al., 1987). Recombinase activity was studied by using a previously described strategy (Bar et al., 1996) in which the effectiveness of FLP was tested by excisional activation of a blocked *gusA* in the target recombination-reporter construct, pFFG (Bar et al., 1996). The binary vector plasmids pJFL, pJFLO and pFFG were introduced into tobacco plants via *Agrobacterium*-mediated leaf disk transformation and hemizygous, single-locus lines were selected (as described below) for further study. To monitor FLP activity through different stages of plant development, JFL or JFLO loci were brought into the same plants as FFG loci, by cross-pollination of each of the four target lines, FFG(601), FFG(605), FFG(611) and FFG(619), with pollen from JFL(74), JFL(201), JFLO(810) and JFLO(811) lines. Recombination activity was monitored both molecularly and phenotypically, by excisional activation of GUS expression in the target FFG locus in the hybrid progenies.

GUS activity, reflecting recombination-activation of *gusA*, was initially measured directly in mature seeds. Bulks of 25 seeds (repeated in at least five consecutive experiments) from each hybrid were surface-sterilized, ground and incubated in 200µl of GUS assay buffer (GAB) at 37°C for 3 h. As indicated by fluorometric assays (Table 2) GUS activity was positively scored in progenies of the JFLO(811) x FFG(601) and JFLO(811) x FFG(611) hybrids only. In contrast, no GUS activity was observed at this stage in crosses of JFLO(811) with FFG(605) and FFG(619) as well as in crosses of JFL(74), JFL(201) and JFLO(810) with all four FFG lines that were tested. Cotyledon samples from hybrid seedling progenies of each of the eight JFL x FFG and eight JFLO x FFG crosses exhibited GUS activity (Table 2), indicating active recombinase and target-construct loci in the corresponding parental lines.

Table 2. GUS activity in JFL x FFG and JFLO x FFG (F₁) hybrid seeds and seedlings

	Seeds ^a		Seedlings ^b	
	tested	GUS activity	Tested	Gus (+)
JFL(74)xFFG(601)	150	(-)	46	9
xFFG(605)	250	(-)	48	13
xFFG(611)	175	(-)	43	9
xFFG(619)	125	(-)	50	12
JFL(201)xFFG(601)	250	(-)	41	11
xFFG(605)	550	(-)	71	19
xFFG(611)	300	(-)	41	8
xFFG(619)	125	(-)	48	11
JFLO(810)xFFG(601)	150	(-)	45	11
xFFG(605)	125	(-)	49	13
xFFG(611)	125	(-)	46	12
xFFG(619)	150	(-)	42	12
JFLO(811)xFFG(601)	375	(+)	41	9
xFFG(605)	550	(-)	41	8
xFFG(611)	250	(+)	48	13
xFFG(619)	250	(-)	44	11

^aMature seeds were tested in bulks of 25 at a time. Positive (+) and negative(-) GUS expression was determined as in Materials and methods.

^bGUS expression was scored in cotyledons of 1-2-true-leaf stage seedlings that germinated in the absence of antibiotics, hence, the expected percentage of GUS(+) seedlings is 25%.

GUS activity in 200-250 seeds and 45-50 seedlings progeny derived from self-pollinated parental lines scored under 0.02 units while pGUS plants scored above 0.28 units.

Crosses of JFL(74) and JFLO(811) with each of the target lines FFG(601) and FFG(611) were further used for a comparative study of timing and efficiency of recombination through somatic and germinal stages.

To test germinal transfer of the recombined locus, two randomly selected F₁ progenies of each JFL(74) x FFG cross and three and four randomly selected GUS(+) progeny of JFLO(811) x FFG(601) and JFLO(811) x FFG(611), respectively, were self-fertilized. Genetic segregation predicts different ratios: 9:7 for GUS(+):GUS(-) if the JFLO and FFG loci were transferred independently and activation occurred in F₂; and 3:1 for GUS(+):GUS(-) if the recombined *35sPΩ-frt-gusA* loci were transferred through germline cells. Additionally, Since no GUS activity was observed in seedlings derived from self-pollination of the FFG lines we conclude that no measurable spontaneous recombination of *frts* occurred in the tobacco genome, therefore, GUS(+) phenotypes in the absence of JFLO would essentially be due to germline transfer of the recombined loci. Inheritance of the recombined locus was therefore tested in GUS(+) F₂ progeny by molecular analysis for the presence of the

JFLO locus. The absence of the JFLO locus, represented by a 0.15-kb PCR product in GUS(+) phenotypes would confirm the inheritance of the recombined locus.

All four JFL(74)-derived progenies showed approximately 9:7 ratio of stained to non-stained seedlings, consistent with previously reported F₂ segregation of JFL x FFG hybrids (Bar et al., 1996). Similarly, three JFLO(811) x FFG lines (F₁-26, F₁-30, F₁-04) segregated in approximately 9:7 ratio (Table 3) consistent with independent segregation of the JFLO and FFG loci. In contrast, four of the seven JFLO(811)-derived F₂ progenies segregated in ratios which are significantly different (at $p > 0.05$) from the 9:7 GUS(+):GUS(-) ratio expected when the recombinase and target loci are transferred independently. Progeny of these lines (F₁-66, F₁-06, F₁-52, F₁-94) segregated in nearly the 3:1 GUS(+):GUS(-) ratio expected when the recombined locus is inherited (Table 3).

Table 3. Segregation of GUS(+) phenotypes in F₂ progeny derived from self-fertilized JFLO(811) x FFG(601) and JFLO(811) x FFG(611) F₁ hybrids

Cross	Hybrid line	Observed numbers of progeny seedlings		
		F ₂ tested	GUS(+)	%
JFLO (811)	F ₁ -26	50	27	54
X	F ₁ -30	82	47	57
FFG (601)	F ₁ -66	82	58	70
JFLO (811)	F ₁ -04	50	29	58
X	F ₁ -06	50	34	68
FFG (611)	F ₁ -52	100	80	80
	F ₁ -94	50	36	72

PCR/Southern blot analysis was used to verify germline inheritance of the recombined loci. PCR of F₂ GUS(+) seedlings with primers specific to the 35sPQ-*FLP* locus following hybridization to *FLP*-specific probe revealed the absence of the 0.15-kb *FLP*-specific product, showing lack of the JFLO locus in nine out of 35 GUS(+) progenies derived from self-pollination of the JFLO(811) x FFG(611) F₁-52 plant (Figure 1, lanes 7, 10, 11 and 12). These molecular results, in conjunction with the expected 3:1 ratio of segregation for presence:absence of the JFLO locus in the F₂ GUS(+) progenies, directly account for germline recombination in high frequency in this line.

The finding of germline transmission in the F₁ hybrids that showed embryonally early recombination, in contrast to the embryonally late recombination hybrids that were studied, raise the possible usefulness of pre-screening of mature seeds as a strategy for selecting lines that facilitate efficient recombination and germinal inheritance of the recombined *frt* loci. A most likely explanation for the association of early recombination and germline transmission would be through activation of recombinase in the embryo shoot meristem cells that give rise to the development of inflorescence germline cells in the F₁ hybrid. In that respect, clonal development of embryonic meristem recombination events would greatly increase the probability of generation of whole-plant recombination patterns and germline transmission of recombined loci.

Appendix D: FLP-mediated restoration of fertility in *rolC* transgenic tobacco

Site-specific recombination systems can function as molecular tools to facilitate deletion of gene expression units in transgenic plants. Here we tested the feasibility of genetic reversion, mediated by FLP recombinase, of *rolC* (*A. rhizogenes*) - induced male sterility in tobacco. The constitutive expression of *rolC*, driven by the cauliflower mosaic virus (CaMV) 35s promoter, induces pleiotropic effects including reduced apical dominance and plant height, lanceolate and pale green leaves and small, male sterile flowers with reduced female fertility (Schmulling et al., 1988).

The FLP recombinase target construct, pFCF, contains a 35sP-*rolC* expression cassette (Spena et al., 1987) that was positioned between directly oriented *frt* sites. The expression of *rolC* from pFCF in tobacco was expected to induce the characteristic *rolC* pleiotropic phenotypes. FCF was designed to serve as a target locus for FLP- excisional recombination activity, thus to enable studying the feasibility of reversion of the *rolC*-induced vegetative and reproductive traits in the FCF x JFLO hybrid progenies in result of excision of the genomic 35sP-*rolC* expression cassette.

The binary vector plasmids pJFLO and pFCF were introduced into tobacco plants via *Agrobacterium*-mediated leaf disk transformation and the hemizygous, single-locus lines, JFLO(811), FCF(107) and FCF(109) were selected for further study. Both FCF lines displayed the characteristic *rolC* pleiotropic phenotypes described above, while JFLO(811) plant phenotypes were normal. Restoration of fertility was studied by the ability of hybrid plants to either self or cross-fertilize wild-type plants. Consistently, seed yield per capsule in self-fertilized FCF, JFLO(811) and wild-type plants was zero, 1760 ± 121 and 1824 ± 118 , respectively, similar to the yields derived from crosses of each with wild-type plants (Table 4).

Among the 20 progeny having a FCF/JFLO genotype, in ten [Table 4, plants F₁(107)-36, 44, 45, 58 and F₁(109)-2, 8, 9, 17, 47, 59] seed yield derived from selfing and out-cross tests ranged between 1485 ± 130 - 1962 ± 172 and 1307 ± 128 - 1817 ± 201 , respectively, not significantly different ($p < 0.05$) from wild-type (1824 ± 118 - 1964 ± 169). These results indicate restoration of both female and male fertility, similar to wild type. The only exception, however, was F₁(109)-17 which after selfing,

Table 4. Seed yield of tobacco wild-type (SNN) plants, FCF maternal parent, JFLO paternal parent and FCF x JFLO self and cross progeny.

Plant no.	Self seed set ²	Cross seed set ²
FCF	0 ^f	0 ^f
JFLO (811)	1760 ± 121 ^a	1724 ± 143 ^a
SNN (WT)	1824 ± 118 ^a	1964 ± 168 ^a
<hr/>		
F ₁ (107) ¹ -		
8	348 ± 139 ^{de}	470 ± 57 ^{de}
17	0 ^f	0 ^f
17/1	541 ± 84 ^{cd}	698 ± 123 ^{cd}
36	1527 ± 187 ^{ab}	1677 ± 191 ^a
38	975 ± 172 ^{bc}	1231 ± 172 ^{ab}
42	807 ± 94 ^{cd}	1020 ± 143 ^{bc}
44	1860 ± 129 ^a	1653 ± 111 ^a
45	1718 ± 172 ^a	1643 ± 166 ^a
58	1514 ± 112 ^{ab}	1728 ± 177 ^a
59	676 ± 94 ^{cd}	811 ± 151 ^{cd}
60	595 ± 152 ^{cd}	675 ± 143 ^{cd}
61	0 ^f	0 ^f
61/1	775 ± 126 ^{cd}	1326 ± 157 ^{ab}
<hr/>		
F ₁ (109) ¹		
1	443 ± 107 ^{cde}	620 ± 65 ^{cd}
2	1862 ± 172 ^a	1689 ± 190 ^a
8	1885 ± 184 ^a	1801 ± 213 ^a
9	1916 ± 174 ^a	1817 ± 201 ^a
17	960 ± 125 ^{bc}	1530 ± 212 ^{ab}
38	628 ± 124 ^{cd}	759 ± 119 ^{cd}
41	135 ± 31 ^e	380 ± 89 ^{de}
47	1485 ± 130 ^{ab}	1711 ± 169 ^a
59	1562 ± 135 ^{ab}	1307 ± 128 ^{ab}

¹F₁(107) and F₁(109) progeny correspond to the crosses of FCF(107) and FCF(109) with JFLO(811), respectively.

²Average number of seed in five seed capsules derived from selfing or cross-pollination of wild-type (SNN) plants; low case letters represent significance differences at p<0.05.

yielded 960 ± 125 seeds per capsule, 53% of wild-type. This very likely reflects reduced female fertility, as pollen fertility was similar to wild type, yielding 1530 ± 212 seed per capsule on an out-crossing test (Table 4). In two additional plants [(Table 4, plants F₁(107)-38,42)] seed yields were 807 ± 94 and 975 ± 172 in selfing, and 1020 ± 143 and 1231 ± 172 in out-cross tests, respectively. This indicates restoration of male fertility to 52-63% of wild-type (1964 ± 169). In additional six progeny [Table 4,

plants F₁(107)-8,59,60 and F₁(109)-1,38,41] seed yields were 7-53% and 19-41% of wild-type, in self and out-cross tests, respectively, while in two progeny [Table 4, F₁(107)-17, 61] male sterile flowers were developed, similar to their corresponding FCF parents.

In these experiments one FLP expressor line was crossed with two FCF target lines. The results indicate large variability in the restored fertility trait among each of the two F₁ progeny populations. Such variability, found among genotypically identical sibs, may rise from differences in methylation (or other epigenetic factors) of the JFLO and FCF genomic loci in different sibs. One approach to obtain an efficient excision reaction and gain uniformity in the recombination-mediated trait would be the pre screening of mature seeds for crosses that facilitate early recombination event, as described in Part B, above. Yet, further studies would be required for the better understanding of these basic questions. This work was recently presented in The 6th International Congress of Plant Molecular Biology, Quebec, Canada (Gidoni et al., 2000).

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