BARD Final Scientific Report

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BARD Project Number: US-3245-01

Project Title: Regulated Expression of Site-Specific DNA Recombination for Precision Genetic Engineering of Apple.

Investigators

Institutions

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Keywords: recombinase, plant_transformation, inducible_promoters, marker_excision, markless_selection, light_inducible_promoter, estrogen_inducible_promoter, *Malus*.

Abbreviations commonly used in the report, in alphabetical order:

DNA = deoxyribonucleic acid, genetic material of cells.

FLP = recombinase protein native to *Saccharomyces cerevisiae*.

FRT = specific DNA recombination target of FLP (site-specific).

GATEWAY = DNA cloning technology that utilizes lambda phage site-specific recombination (® Invitrogen).

GUS = β -glucuronidase.

PCR = polymerase chain reaction.

TOPO = DNA cloning technology where the enzyme topoisomerase I is used in place of DNA ligase (® Invitrogen).

uidA = gene encoding β -glucuronidase (GUS).

XVE = DNA-binding domain of the bacterial repressor LexA (X), the acidic transactivating domain of VP16 (V), and the regulatory region of the human estrogen receptor (E).

Budget: IS: \$152,000

US: \$153,000

Total: \$305,000

Signature
Principal Investigator

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Authorizing Official, Principal Institution

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)				0
Submitted, in review, in preparation	3			3
Invited review papers				0
Book chapters				0
Books				0
Master theses				0
Ph.D. theses				0
Abstracts				0
Not refereed (proceedings, reports, etc.)				0

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

NONE

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings		2	1	3
Longer Visits (Sabbaticals)				0

Description of Cooperation:

Evaluation and development of inducible promoters for the expression of recombinase in apple was conducted at USDA-ARS. Marker free apple transformation methods were developed at Cornell University. The activity of the FLP/*FRT* recombinase system in apple was determined at ARO and the use of the FLP recombinase system under light-inducible expression in apple was then developed at the ARO.

Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
Submitted				0
Issued (allowed)				0
Licensed				0

ABSTRACT

Title: Regulated Expression of Site-Specific DNA Recombination for Precision Genetic Engineering of Apple.

Objectives: The original objectives of this project were to: 1) evaluate inducible promoters for the expression of recombinase in apple (USDA-ARS); 2) develop alternative selectable markers for use in apple to facilitate the positive selection of gene excision by recombinase (Cornell University); 3) compare the activity of three different recombinase systems (*Cre/lox*, *FLP/FRT*, and *R/RS*) in apple using a rapid transient assay (ARO); and 4) evaluate the use of recombinase systems in apple using the best promoters, selectable markers and recombinase systems identified in 1, 2 and 3 above (Collaboratively). Objective 2 was revised from the development alternative selectable markers, to the development of a marker-free selection system for apple. This change in approach was taken due to the inefficiency of the alternative markers initially evaluated in apple, phosphomannose-isomerase and 2-deoxyglucose-6-phosphate phosphatase, and the regulatory advantages of a marker-free system. Objective 3 was revised to focus primarily on the *FLP/FRT* recombinase system, due to the initial success obtained with this recombinase system. Based upon cooperation between researchers (see Achievements below), research to evaluate the use of the *FLP* recombinase system under light-inducible expression in apple was then conducted at the ARO (Objective 4).

Background: Genomic research and genetic engineering have tremendous potential to enhance crop performance, improve food quality and increase farm profits. However, implementing the knowledge of genomics through genetically engineered fruit crops has many hurdles to be overcome before it can become a reality in the orchard. Among the most important hurdles are consumer concerns regarding the safety of transgenics and the impact this may have on marketing. The goal of this project was to develop plant transformation technologies to mitigate these concerns.

Major achievements: Our results indicate activity of the *FLP/FRT* site-specific recombination system for the first time in apple, and additionally, we show light-inducible activation of the recombinase in trees. Initial selection of apple transformation events is conducted under dark conditions, and tissue cultures are then moved to light conditions to promote marker excision and plant development. As trees are perennial and cross-fertilization is not practical, the light-induced *FLP*-mediated recombination approach shown here provides an alternative to previously reported chemically induced recombinase approaches. In addition, a method was developed to transform apple without the use of herbicide or antibiotic resistance marker genes (marker free). Both light and chemically inducible promoters were developed to allow controlled gene expression in fruit crops.

Implications: The research supported by this grant has demonstrated the feasibility of “marker excision” and “marker free” transformation technologies in apple. The use of these safer technologies for the genetic enhancement of apple varieties and rootstocks for various traits will serve to mitigate many of the consumer and environmental concerns facing the commercialization of these improved varieties.

ACHIEVEMENTS

Main scientific achievements:

Objective 1: evaluate promoters for inducible gene expression in apple.

A chemically inducible promoter (XVE) previously developed for use in *Arabidopsis* and tobacco, was modified for efficient use in apple. The modified XVE promoter uses a GATEWAY cloning site for insertion of the gene-of-interest and was found to result in higher levels of gene expression than when gene insertion was conducted by standard PCR or TOPO cloning methods. The chemical-inducible XVE promoter and the light-inducible CAB promoter were transferred to apple to evaluate their expression in apple.

Objective 2: develop marker-free transformation methods for apple.

A technique was developed to transform apple without the use of a selectable marker. The procedure was applied with success to two different genotypes of apple, Galaxy and M.26, with a transformation efficiency of 12% and 25%, respectively. Because this technology does not involve the use of antibiotic resistance genes or other marker genes with negative connotations, it will eliminate a source of consumer and activist concern, and will simplify the regulatory process. As far as we know, the results obtained (see Appendix) are unprecedented.

Objective 3 and 4: evaluate activity of FLP/FRT recombinase system in apple.

To determine the recombination activity of FLP in apple, a transgenic line of the apple cultivar 'Galaxy' was generated using a vector that constitutively expressed FLP. Activity of FLP recombinase in apple cells was demonstrated by a gain-of-function assay that resulted in GUS activity following excision of the antibiotic resistance gene used as a selectable marker (see Appendix). A similar gain-of-function assay was also used to demonstrate regulated FLP expression in apple. To achieve regulated expression, a light inducible (chlorophyll *ab* binding protein gene) CAB-promoter was used, and to prevent FLP expression in bacterial cells, a plant intron was introduced at the 5' untranslated region of *FLP*. The light-induced FLP-mediated recombination approach achieved by this research provides an alternative to the previously reported chemical induction approaches, and can potentially be used for excision-manipulation of transgenes to generate marker-free and recombinase-free trees.

ACHIEVEMENTS

Details of cooperation:

The research conducted during the three year project was carried out in full cooperation between the two Israeli and the two American laboratories. All four investigators participated in a conference call at the start of project to develop a plan to facilitate interaction and cooperation in completing the goals of the project. The investigators decided to rely primarily on email to maintain cooperative interaction, supplemented with meetings between research groups whenever possible. Emails were exchanged as needed to confer on research needs and developments.

M. Flaishman (ARO) and D. Gidoni (ARO) met on a regular basis to discuss and plan for the work conducted by their teams in Israel. J. Norelli (USDA-ARS) and H. Aldwinckle (Cornell University) communicated regularly by telephone and email, and J. Norelli visited the Aldwinckle lab on December 10, 2002 and on May 18, 2004 to discuss research results and plans. One member of the Norelli lab spent one week working in the Aldwinckle lab from January 27-31, 2003, and two members of the Norelli lab spent one week working in the Aldwinckle lab from October 18-22, 2004. DNA vectors were exchanged between the Norelli program and the Aldwinckle program, and transgenic plants were created by the Aldwinckle program to facilitate evaluation of inducible promoters by the Norelli program.

H. Aldwinckle (USA) and M. Flaishman (Israel) met in August 2002 at the XXVI International Horticultural Congress (Toronto). H. Aldwinckle (USA), J. Norelli (USA) and M. Flaishman (Israel) met in Geneva, NY on July 28-30, 2003, to discuss research results and determined the best approach for completing Objective 4. On May 21, 2004, H. Aldwinckle (USA) and D. Gidoni (Israel) met in San Francisco and conducted a conference call with J. Norelli (USA) to discuss the project's progress and conclusion, and potential joint publication of the results.

ACHIEVEMENTS

List of publications where BARD is acknowledged to date:

Gidoni D, Bar M, Leshem B, Gilboa N, Mett A and Feiler J (2001a) Embryonal recombination and germline inheritance of recombined *FRT* loci mediated by constitutively expressed FLP in tobacco. *Euphytica* 121: 145-156.

Gidoni, D, Bar M and Gilboa N (2001b) FLP/*FRT*-mediated restoration of normal phenotypes and clonal sectors formation in *rolC* transgenic tobacco. *Transgenic Res.* 10: 317-328.

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

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NOTE: Manuscript in preparation will be sent to BARD when they are submitted for publication and three reprints will be sent upon publication.

Unpublished data: Objective 2

Genetic Transformation of Apple Without Use of a Selectable Marker

Reported by H. S. Aldwinckle

Mickael Malnoy and Ewa Borejsza-Wysocka contributed greatly to this project.

Selectable marker genes are widely used for the efficient transformation of crop plants. In most cases, selection is based on antibiotic or herbicide resistance. These marker genes are preferred because they tend to be most efficient (e.g. in apple up to 80% transformation). Due mainly to consumer and grower concerns, considerable effort is being put into developing a suite of strategies (site-specific recombination, homologous recombination, transposition and co-transformation) to eliminate the marker gene from the nuclear or chloroplast genome after selection. Current efforts concentrate on systems where the marker genes are eliminated efficiently soon after transformation. However these methods are multi-step and must be refined to achieve 100% marker elimination. Alternatively, transgenic plants can be produced by use of marker genes that do not rely on antibiotic or herbicide resistance but instead promote regeneration after transformation. Examples are phosphomannose isomerase, xylulose isomerase, β -glucuronidase or isopentenyl transferase. However these methods are generally of low efficiency.

For the commercialization of transgenic plants use of an efficient marker-free technology would be desirable, since there would be no involvement of antibiotic resistance genes or other marker genes with negative connotations. This would eliminate the marker gene as a source of consumer and activist concern, and would simplify the

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regulatory process. With this goal in mind, we have worked to develop a technique for apple transformation without use of a selectable marker.

For testing of the feasibility of apple transformation without any selectable marker, two binary vector constructs, pPin2mpNPR1 and pWiATT-35Suida, without the selectable marker (nptII gene) has been created. Transgenic apple lines were obtained using *Agrobacterium* mediated transformation. All the regenerant growing in the media without selectable marker were transferred to an individual tube and tested by PCR to confirm the presence of the gene of interest. Two genotypes of apple (M.26 and Galaxy) were initially employed for this procedure.

Two transformations of the apple rootstock M.26 with the binary vector pin2MmpNPR1 without the kanamycin resistance gene were completed. 1000 regenerants for each transformation were harvested from leaf-piece transformation plates. 300 and 250 of the regenerants were chosen randomly and tested by PCR for the presence of the pin2 promoter (Figure 1). Seventy-six (25.4%) and fifty-five (22%) of these regenerants showed integration of the pin2 promoter. The parent M.26 was negative for pin2 by PCR.

Figure 1: PCR analysis of the putative transgenic M.26 pin2mpNPR1 kan (-) lines with the primer pin2 Forward and Reverse. N: negative control; +: Plasmid pin2mpNPR1 kan (-).

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A Southern analysis will also be carried out for additional confirmation of transformation.

A second transformation experiment of M.26 with the binary vector pWiAtt 35SuidA (GUS) was performed and 1500 regenerants were harvested from leaf-piece transformation plates. 566 regenerants were chosen randomly and tested by GUS staining for the presence of the *uidA* gene. One hundred forty five (25.5%) of the regenerants were positive for the presence of *uidA* (Figure 2).

Figure 2: GUS histochemical test on microshoots of M.26, M.26 35S *uidA* and putative transgenic M.26 pWiATT 35S *uidA*

One hundred lines positive for GUS staining and 50 lines negative for GUS staining were selected and propagated. These lines will be tested by PCR with the primers for *att* and *uidA* to confirm the integration of the T-DNA.

These data showed that transformation and selection of M.26 without use of a selectable marker is possible. These results showed also that a consistent transformation rate of ca. 25% rate for different transformations with the same or different binary vectors.

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One transformation experiment with the apple genotype Galaxy with the binary vector pin2mpNPR1 without the kanamycin resistance gene was done. 1300 regenerants were harvested from leaf-piece transformation plates. One hundred fifty eight of the regenerants were tested by PCR for the presence of the pin2 promoter. Nineteen (12%) of these regenerants showed integration of the pin2 promoter. The parent Galaxy was negative for pin2 by PCR.

CONCLUSIONS

These results showed that we have been able to develop a technique for apple transformation without using a selectable marker. This procedure was applied with success to two different genotypes of apple, Galaxy and M.26, with a transformation efficiency of 12% and 25%, respectively.

Although the technique appears relatively simple for anyone familiar with plant transformation techniques, the results we have obtained are unprecedented for fruit crops as far as we know.

In summary, we have good preliminary evidence that markerless transformation of apple can be done in a relatively efficient fashion. The efficiency seen so far is certainly high enough to use on a commercial scale for engineering apple varieties and rootstocks for various traits.

Unpublished data: Objective 3-4

Research activities at the ARO, Bet Dagan, Israel

Reported by D. Gidoni and M. Flaishman

Constitutive and light-induced FLP-mediated recombination in apple

For testing of the feasibility of FLP recombination activity in apple, we generated a transgenic line of apple Galaxy cultivar using FLP-containing binary vector construct pJFLO (Gidoni et al., 2001). In pJFLO, FLP is expressed constitutively under the CaMV 35S promoter (Figure 1). Transgenic apple was obtained using *Agrobacterium*-mediated transformation followed by selection for resistance to kanamycin, and the presence of the gene was confirmed by PCR, using primers specific to the JFLO locus (Gidoni et al.,

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2001). In order to allow FLP-mediated recombination, a recombination-reporter construct, pFFG (Bar et al., 1996) was transiently introduced into transgenic JFLO explants, using *Agrobacterium*-mediated transformation. In the pFFG construct, the presence of a 1.31 kb blocking DNA fragment (*npt*) flanked by directly oriented *FRT* sites between the CaMV 35S promoter and the downstream *gusA* coding region prevents *gusA* transcription. Excision of the blocking sequence by FLP brings together the 35S promoter and the downstream *gusA* reporter gene, giving rise to *gusA* expression.

pJFLO binary vector cassette: LB-----35S- Ω -FLP -----RB

pFFG binary vector: LB----35S- Ω -FRT---NPTII ("block")---FRT- gusA----RB

Figure 1: Schematic presentations of pJFLO and pFFG binary vector plasmids (Gidoni et al., 2001, Euphytica, 121:145).

After introduction of pFFG into JFLO explants, plant tissue was stained and displayed sectors of GUS activity (Figure 2). These results demonstrate the activity of FLP recombinase in apple cells.

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Figure 2: Presentation of FLP-mediated GUS activity sectors in transgenic FLP expressing apple Galaxy leaves, after *Agrobacterium*-mediated transient introduction of the pFFG recombination-substrate vector.

In order to develop regulated FLP expression in apple, we (in the DG lab.) constructed pCFFG (Figure 3). In this binary (pCAMBIA) vector construct, a light inducible (chlorophyll ab binding protein gene) Cab-promoter - *FLP* and an *NPTII* expression unit were placed between *FRT* sites, and a *gusA* reporter gene was placed downstream of the right *FRT*. In order to prevent FLP expression in bacterial cells, a plant intron was introduced at the 5' untranslated region of *FLP*. An induced FLP-mediated recombination after plant transformation, is expected to excise both the *FLP* and *NPTII* genes, giving rise to expression of *gusA* reporter.

pCFFG: LB---light inducible promoter-*FRT*-*FLP*- *NPTII*---*FRT*-*gusA*---RB

Figure 3: A schematic presentation of pCFFG binary vector plasmid, harboring the FLP recombinase gene under the chlorophyll ab binding protein (Cab) gene promoter of petunia (Gidoni et al., 1988, *Mol. Gen. Genet.* 211: 507-514).

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In order to test this hypothesis, we generated (in the MF lab.) 20 transgenic lines of apple Galaxy cultivar using the Cab promoter-FLP-containing binary vector construct pCFFG. Transgenic apple seedlings were obtained using *Agrobacterium*-mediated transformation and selection for resistance to kanamycin during the callus stage in the dark, followed by regeneration of shoots under light conditions. The presence of the gene was confirmed by PCR, using primers specific to the *FLP*, *NPTII* and *GUS* loci (Gidoni et al., 2001, *Euphytica* 121: 145-156).

GUS activity assays, as confirmed by PCR analyses of leaf tissue from these plants, indicated sectorial patterns of somatic (late) recombination activity in one plant (Gal-127). These patterns remained in the lateral shoots after cuttings, indicating that recombination occurred in somatic, however not in shoot meristem cells.

In order to generate plants with 100% recombination, namely, completely excised of *NPTII* and *FLP* expression cassettes, regeneration of GUS-positive whole plants from leaf tissue is currently in progress. Potentially recombination-positive two additional lines (Gal-128 and Gal-130) are being currently tested.

The light-induced FLP-mediated recombination approach shown here, provides an alternative to the previously reported chemical induction approaches, with the R/RS system by Sugita et al. (2000) and with the Cre/*lox* system by Zuo et al. (2001) which can potentially be used for excision-manipulation of transgenes, more particularly, generation of selectable marker-free and recombinase-free trees.

References

Bar M, Leshem B, Gilboa N and Gidoni D (1996) Visual characterization of recombination at *FRT-gusA* loci in transgenic tobacco mediated by constitutive expression of the native FLP recombinase. *Theor Appl Genet* 93: 407-413.

Gidoni D, Bar M, Leshem B, Gilboa N, Mett A and Feiler J (2001a) Embryonal recombination and germline inheritance of recombined *FRT* loci mediated by constitutively expressed FLP in tobacco. *Euphytica* 121: 145-156.

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vector for the production of marker-free transgenic plants containing a single copy transgene at high frequency. Plant J 22: 461-469.

Zuo J, Niu Q-W, Moller SG and Chua N-H (2001) Chemical-regulated, site-specific excision in transgenic plants. Nature Biotechnol 19: 157-161.