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FINAL REPORT

**Exploration of new perspectives and limitations in *Agrobacterium*-mediated gene transfer technology.(#DE-F602-92ER20073-**

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Genetic manipulation of plants often involves the introduction of homologous or partly homologous genes. Ectopic introduction of homologous sequences into plant genomes may trigger epigenetic changes, **making expression of these genes unpredictable.** Expression of randomly introduced non-homologous transgenes is also unpredictable because of the so called position effect. Random insertion of transgenes is also mutagenic for the recipient cells. Problems caused by random integration of the transforming sequences could be avoided by **homologous gene targeting (HGT).** The main objective of this project, therefore, was to examine the feasibility of using ***Agrobacterium*-mediated gene transfer (AMGT)** for HGT in plants.

CONCISE SUMMARY OF OUR PROGRESS

1. Identification and preliminary characterization of AMGT-related mutagenesis, which seems to be the result of a process analogous to bacterial error prone repair.
2. Discovery of frequent, long T-DNA transfer, which can be utilized for introducing extreme long T-DNA-s required for the multiple negative selection based HGT.
3. Development of the *Arabidopsis* tissue culture and cell genetic background for molecular biology such as totipotent sustained root cultures, protoplast cultures and facile transformation.
4. Completion of the **core *Nia2* homologous gene targeting vectors.**
5. AMGT experiments using vector pMXY16 yielded a large number of transgenic plants which are being analyzed. Ectopic introduction of the *Nia2* sequences led us to recognize a system where full gene silencing can be obtained in nearly all of the transformants.
6. Factors involved in gene silencing can be identified in this easy test system. 5' and 3' truncations of the *Nia2* gene and inversion of the nested selectable marker in plasmid pMXY16 have been done to test the effect of modified transcription and mRNA stability.
7. New negative selection markers and their characterization at the level of transformation.
   a. Domestication of diphtheria toxin A gene by seed-specific expression under control of the vicilin promoter, and identification of a transient inhibitory effect on transformation.
   b. Development of the HSVtk conditional lethal negative selection marker for transformation.
   c. Development of *Pseudomonas* and *Rhodococcus* aromatic oxidases for negative selection.

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d. Development of an antisense hygromycin resistance gene conditional positive selection.

8. New HGT approach based on the homology search by the extreme left side of the T-region during T-DNA integration. Construction of binary vectors with left border homology to *Nia2* gene.

DETAILED SUMMARY OF OUR PROGRESS

**1. Transformation related mutagenesis and homologous recombination**

In the *Nicotiana plumbaginifolia* system where we recognized AMGT related mutagenesis two putative homologous recombinants could not be characterized completely because of the inappropriate genetics of the recipient system. In these lines the target sequence was not seen but a "recombinant" fragment appeared, although not in the original sequence environment. Similar results were obtained with tobacco (Offringa et al. 1993) where **non-reciprocal homologous recombination** resulted in a subsequent (distant) integration of a recombinant fragment. The target remained virtually intact. In our lines the chromosomal location could not be established genetically because of sterility. Also the somatic hybridization and segregation approach could not be used because a genetic map is unavailable.

**2. Long T-DNA transfer**

Analysis of recombination-like integration patterns of transformants revealed a very important new feature of the AMGT process: frequent "long T-DNA transfer". T-DNA transfer was not terminated by the left border repeat leading to transfer and integration of the entire binary vector molecule into the plant genome. Since every component of the vector hybridized to the same large genomic fragments in Southern blot experiments, we concluded that the integrated long T-DNA is present as a contiguous piece. Although the capability of *Agrobacterium* to transfer extremely long T-DNAs in the absence of proper left-border was reported (Miranda et al 1992), the frequency (3 out of 5 transformants) of spontaneous long transfer in our system was surprisingly high. These data show that the transfer of long, complex T-DNAs such as those targeting whole genes is quite feasible using AMGT. It will allow us to introduce very long T-DNAs into the plants. (Czako M et al. submitted to *Plant Physiol*, see appendix).

**This observation encouraged us to construct gene targeting vectors with long T-DNA-s (with several markers) since the T-DNA size does not seem to be a limiting factor in the transfer process.**

**3. Sustained root culture and root derived suspension culture of a hemizygous *NIA2* mutant, G-5**

The recipient plants for the T-DNA transfer experiments were the G5 x wild-type F1 hybrid plant 'G5RS' and the wild type control. The *G5* mutant of *Arabidopsis* exhibits reduced NR-activity (yet suffers no growth disadvantage) and as a consequence, resistance to chlorate. G5 (kindly provided by Dr. Nigel Crawford for these experiments) is homozygous for a 25 kb deletion (*chl3-5* allele; Wilkinson and Crawford 1991, 1993) which completely eliminates the major NR apoenzyme gene. G5RS is heterozygous for *chl3-5*, i.e., hemizygous for the *NIA2* gene, providing a single target for HGT. The otherwise diploid genetic constitution excludes

the manifestation of other recessive chlorate resistance mutations, i.e., those not affecting the *NIA2* gene. The other known recessive chlorate resistance mutations are either on different chromosomes (*chl2* and *cnx*) or do not show high linkage (*chl1* on chromosome 1; Doddema *et* al. 1978; Koorneef *et* al. 1983; Koorneef 1990 - *NIA1* gene, Cheng *et* al. 1988). In addition to the *NIA2* gene, the RLD genotype provided the extremely efficient regeneration and transformation competence. Therefore, the transfer of the *NIA2* deletion into RLD background is in progress. A final cross with Columbia WT gives the hemizygote. G5RS is being maintained as a sustained root culture which can be transformed directly (Czako et al. 1993) or it can be first converted into a totipotent cell suspension culture (Mathur et al. 1995), which gives transformants at very high frequency, up to 80% of plated colonies (Columbia ecotype, Wenck, unpublished).

The root culture **is fully totipotent** and genetically stable in long term culture and/or storage). These tools are all very important prerequisites for a homologous gene targeting recipient system utilizing a heterozygous plant. In addition, the embryogenic green mass regenerating from these root cultures proved to be a high yield source of **totipotent protoplast cultures**. More than 60% of the protoclones regenerated multiple shoots (Wenck and Marton, 1995).

## 4. Construction of homologous gene replacement vectors and transformation of the *NIA2* hemizygote recipients.

The core gene targeting vector, pMXY-16 (Fig. 2, in appendix) was constructed from the 5 kb *Sac*I fragment of the *Arabidopsis NIA2* gene (pAt60, Crawford et al. 1989) inserted into pGA768 (pGA768, G. An, unpublished). The kanamycin resistance gene cassette (*nptII*, from pGA472, An et al. 1986) was inserted into the unique *Xho*I site 100 bp downstream of the *NIA2* translation start site. The resulting construct, pMXY-16, contained a 2 kb *NIA2* fragment near the right border of T-DNA and a 3 kb *NIA2* fragment near the left border. The TATA box and the translation start codon as well as the introns are shown in the map. Transcription of *nptII* is in reverse orientation to *NIA2* (Fig. 2). Plasmid pMXY-18, a control vector, contained only *nptII* between the T-region borders.

The ectopic introduction of *NIA2* sequences (by the pMXY-16 vector) caused extremely frequent **silencing of the** *NIA2* gene copy in the transgenic plants (Table 1, appendix). **More interestingly, RLD** cultures, where the ectopic copy of *NIA2* interacts with the (normally at least) two cognate copies, produced only about 3% NR deficient mutants among the kanamycin resistant transgenic plants. This mild cosuppression is in contrast with the extreme, nearly complete (91%) cosuppression observed in the hemizygotes indicating that it is related to the relative copy numbers of the incoming and cognate genes and to the gene replacement construct itself. Identification of the *NIA2* regions responsible for this silencing is part of another ongoing project.

## 5. Negative selection using chimeric marker genes and their incorporation into the homologous gene targeting vector

In order to circumvent the need for selection for chlorate resistance, which is of dubious value due to the frequent silencing, we are going to capitalize on multiple negative selection markers.

**a.** We have identified conditions for negative selection using the **Herpes Simplex Virus thymidine kinase gene** and ganciclovir (St. Clair *et* al. 1984). **At least two orders of magnitude** difference was seen when appropriate culture conditions and optimal timing were used for negative-positive selection in the *Arabidopsis* root transformation system (see attached publication, Czako and Marton 1994. and Czako et al 1995 in press).

**b. The feasibility** of negative selection using the chimeric marker, the **diphtheria toxin A fragment gene**. The presence of a functional **DTxA** gene in a plant transformation vector **reduced the transformation** frequency by several orders of magnitude both in tobacco leaf segments and suspension cells, when it was under the control of the strong, cauliflower mosaic virus 35S promoter **Tight control of *DTxA* expression is possible by developmentally regulated promoters**. Under control of the seed specific **vicilin promoter**, *DtxA* gene does not interfere with normal plant development until after pollination and early embryogenesis (Czako et al. 1992).

**c. The chimeric *Rhodococcus* indole oxidase (CRIO) constructed in our laboratory provided very stringent non-conditional** selection **with no escapees** in *Arabidopsis* root culture transformation experiments. **Not a single** functional transgenic plant could be obtained from root cultures ,where **normally 400-4000** plants can be obtained (Wenck A. Czako M. Marton L: *Rhodococcus* indole oxidase as a negative selection marker in *Arabidopsis* and tobacco, submitted to *Plant J*,

The homologous gene targeting vector pMXY-39 carries the *HSVtk* cassette at the right border (RB), and the seed-specific *DTxA* cassette at the left border.

**d. Development of an antisense hygromycin resistance** gene conditional positive selection.

8. **New HGT approach based,** on the homology search by the extreme left side of the T-region during T-DNA integration has been initiated. Construction of binary vectors with left border homology to *Nia2* gene has been completed.

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