Project Number: 96-DG1

GENETIC ENGINEERING OF 'NONPAREIL' ALMOND

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This project is aimed at developing genetic engineering technologies for the improvement of the almond cultivar 'Nonpareil'. Specifically this proposal aims at the development of shoot regeneration and gene transfer techniques that will permit the introduction of novel genes into almond. Genetic engineering is a powerful new approach that will enhance the ongoing breeding program as it would permit very precise genetic improvement of a variety such as "Nonpareil' with proven commercial potential. The long term goals of this proposal are to provide pest resistance and to reduce/eliminate self-incompatibility. Pest resistance against navel orangeworm is being developed through the introduction and expression of a synthetic gene encoding a *Bacillus thuringiensis* (Bt) insecticidal crystal protein (ICP).

Regeneration experiments with Nonpareil: The focus of all regeneration experiments was on the almond cultivar "Nonpareil" that was introduced into culture last year. Regeneration is the process of obtaining plants from pieces of plant tissue (plant explants). We have focused our regeneration experiments on leaf explants. Leaf tissue is harvested from shoots of Nonpareil grown in culture. These leaves are segmented transversely into strips 2 to 3 mm wide. These strips are then placed on regeneration media. What we have observed to occur on these strips is the formation of adventitious buds The adventitious buds elongate to form shoots which then are multiplied as shoots for propagation or the shoots are induced to form roots to generate plants. We have examined all these processes in the lab over the past year and here are some of our important observations and results.

We observed a better response to the hormone treatments with the *in vitro* cultures this year as compared to last year. This observation is not unusual as plant material stays in culture it tends to become more juvenile and more responsive to plant hormones. And we were able to further confirm observations that we had made with the material last year using different hormone concentrations. The medium used for regeneration was a MS medium (Murashige and Skoog) containing IBA (0.5 mg/L), BA(2-3mg/L) and TDZ (1-3 mg/L). Different permutations containing variations in the concentration of these three hormones was evaluated with respect to the frequency of regeneration as judged by the number of adventitious buds and shoots formed. Last year we observed regeneration occurring in about 10-14 % of the explants. With the changes in the levels of hormones this year we were able to increase the incidence of regeneration only slightly to 14-28%.

Additionally, this year we were also able to evaluate the ability of the regenerated shoots to micropropagate *in vitro*. This step is crucial as we use it to maintain the shoot population in the laboratory. The regenerated shoots can be multiplied with some difficulty depending mainly upon the quality of the regenerated shoots. We observed that about 50-60% of the regenerated shoots can be micropropagated successfully. Rooting of the shoots to obtain plants appears to be a major limiting area too. Rooting was carried out in two steps; first the shoots were placed into a root induction medium (RI), this medium was MS media containing IBA (2-3 mg/L) for 2 weeks. Then the shoots were transferred to a root elongation medium (RE) which consisted of full strength MS medium without any hormones. We observed rooting response in only 20% of the shoots. The response varies from 1-4 roots emerging from the base of the shoots. We have not had the opportunity to test these plants further in the green house or field and this may be necessary to do in the near future when we have this step of the regeneration working well.

Transformation of leaf segments: Leaf explants used for the transformation experiments were harvested from both *in vitro* micropropagated shoots and plants. With a majority of experiments being conducted with leaf explants coming from micropropagated shoot clusters. This is mainly due to rooting problems. In apple transformation we have observed that leaf segments obtained from plants regenerated better that those obtained from shoots. Again like explained above for the regeneration experiments the harvested leaves were cut into strips (transverse, 2-3 mm in with). The leaves are cut directly in the *Agrobacterium* culture. One *Agrobacterium* vector (Fig 1) was used in all experiments and this was EHA101/pDU92.710 that contains three genes; APH encoding resistance to the antibiotic kanamycin for genetic selection of the transformed cells; GUS encoding and enzyme (β-glucuronidase) that permits a rapid screening of transformed tissues and CryIAc a gene from *Bacillus thuringiensis* (Bt) that has insecticidal properties and would provide resistance to navel orangeworm. The bacteria are grown in bacterial medium (523 medium) for 16 to 36 hrs and then diluted with plant media used for regeneration to achieve a final cell concentration of 10^8 cells/ml.

After inoculation of the leaf explants with the *Agrobacterium* vector the explants are allowed to cocultivate for 3-4 days on regeneration medium without any antibiotics to allow infection and transformation to take place. At the end of the cocultivation period the explants were then transferred to a selection media which is regeneration media described above but supplemented with kanamycin (KAN; 50 µg/ml) to select for transgenic tissues and cells and cefotaxime (CEF; 200 µg/ml) to kill specifically the *Agrobacterium* cells. Many thousands of explants have been examined but transformed shoots have not as yet been observed. We consistently see callus which is the first stage. Callus

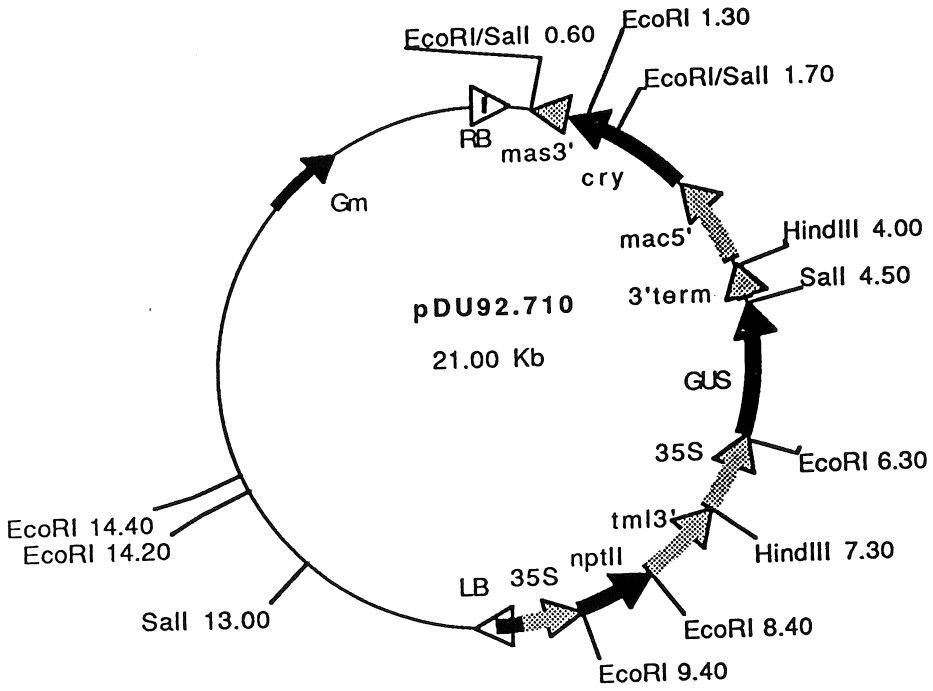
formation can be seen on about 40-50% of the explants. And most of the callus that have been tested show strong GUS activity indicating transformation is occurring. The block seems to be in regenerating these calli to form buds/shoots

Conclusion and future directions: We can conclude from these experiments that transformation is indeed taking place, that genetic selection is working at least at the level of callus formation. We have not been able to regenerate any shoots from callus tissue. It would seem that the tissue being transformed is incapable of regeneration. In a broader sense regeneration is the most significant problem and major hurdle to obtain transgenic almond. More experiments are planned to investigate ways around this problem. Perhaps trying alternative approaches for regeneration through somatic embryogenesis may be of value. Another possibility that we are investigating is altering the concentrations of kanamycin to see if lower concentrations would permit some regeneration and then to determine the percent of the regenerating population that are transformed. Cefotaxime would also need to be investigated to see if it is interfering with regeneration. Additional experiments will also need to be done to increase the frequency of regeneration to at least 80% and above. An increase in the frequency of regeneration can lead to the increase in the frequency of transformation. In different apple cultivars this was most certainly the case with the most regenerable giving the most number of transformants. Different *Agrobacterium* host strains may also need to be tested to find some that do not turn 'on' the almond defense response. The defense response in plants provides the means to stop a pathogen attack and to lead to cell death (apoptosis; programmed cell death). The induction of apoptosis among the cell population in the leaf explant in response to *Agrobacterium* could further limit the regeneration process.

Milestones Achieved:

Developed clean shoot cultures of 'Nonpareil'
Regeneration of untransformed tissue
Micropropagation of *in vitro* shoots
Rooting of micropropagated
Detection of transformed sectors
Development of stable transformed callus

Fig. 1. Physical map of the pDU92.710 vector for the introduction of insect pest resistance in Nonpareil almond.



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March 28, 1997

Dear Chris,

Enclosed please find a copy of a final report on our project (Project No. 96-DG1) entitled "Genetic engineering of 'Nonpareil' almond".

Thank you for your support.

Sincerely,

Abhaya M. Dandekar
Assoc. Prof.