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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 to reduce/eliminate self-incompatibility.

Regeneration experiments with Nonpareil: The focus of all regeneration experiments was on the almond cultivar "Nonpareil". 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 marginal response to the hormone treatments with the *in vitro* cultures this year as compared to last year. We were unable to improve the regeneration beyond the 14-28% rate previously observed. However, for micropropagation this worked fine and we have been able to efficiently micropropagate Nonpareil. We were able to reproduce last years observation with about 50-60% of the regenerated shoots being micropropagated successfully. Rooting of the shoots to obtain plants still remains another major limiting area. However, budding to rootstocks is efficient with the micropropagated cultures and can be used as a route to market this cultivar.

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. The leaf segments were infected with the *Agrobacterium* vector 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. 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.

Identification of stylar Rnases associated with self-incompatibility in almond: Stylar proteins of 13 almond (*Prunus dulcis*) cultivars with known *S*-genotypes were surveyed by IEF and 2D-PAGE combined with immunoblot and N-terminal amino acid sequence analyses to identify *S*-RNases associated with gametophytic SI in this plant species. RNase activities corresponding to *Sa* and *Sb*, two of the four *S*-alleles tested, were identified by IEF and RNase activity staining. The *Sa*-RNase band reacted with the anti-S_4-serum prepared from Japanese pear (*Pyrus serotina*) while no reaction with the antiserum was observed with the *Sb*-RNase band. When the *Sa*-RNase band was excised from IEF gel stained for RNase activity, subjected to SDS-PAGE, and detected by immunoblotting, it appeared that the *Sa*-RNase band consisted of a single protein that reacted with the anti-S_4-serum with *Mr* of about 28 kDa. With 2D-PAGE and silver staining of the stylar extracts, all the four *S*-proteins could be successfully distinguished from each other in highly basic zone of the gel. Although *Sb*-, *Sc*-, and *Sd*-proteins had roughly the same *Mr* of about 30 kDa, *Sc*-protein seemed to be slightly smaller than *Sb*-protein and slightly larger than *Sd*-protein. In 2D-PAGE profiles also, *Sa*-protein had an *Mr* of about 28 kDa and apparently smaller than the other three proteins. A bud sport, in which one of the two *S*-alleles of the original cultivar is impaired, visualized as a loss of *Sc*-protein, which is consistent with the previous pollination study. Interestingly, all the four *S*-proteins reacted with the anti-S_4-serum probably because of the different conformation of *S*-proteins in IEF and 2D-PAGE gels. Having reactivity with the anti-S_4-serum and the same *Mr*, *Sa*-protein in 2D-PAGE appeared to be identical to *Sa*-RNase in IEF and has RNase activity. N-terminal amino acid sequence analysis of the four *S*-proteins revealed that they were highly homologous to each other and similar to *S*-RNases of *Malus*, *Pyrus*,

Scrophulariaceae, and Solanaceae. Taken together, RNases in the style are strongly suggested to be associated with the gametophytic SI of almond. This is the first report identifying and characterizing *S*-RNase in almond.

Conclusion and future directions: We can conclude from these experiments that we need to reevaluate the transformation cultures and protocol with better genes like GFP (Green Fluoroscent Protein). We plan to do that this year. In a broader sense regeneration remains the most significant problem and major hurdle to obtain transgenic almond. Alternate strategies will need to be investigated both in transformation and regeneration using GFP. We need to clone the cDNA encoding the 4 almond S-RNases. Once these are cloned then binary vectors to express antisense will be constructed. Transformation experiments will then be done with these to obtain self-compatible almond cultivars.

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