Project Number: 98-AD-o0

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'. These technologies will be used in an effort to reduce or eliminate self-incompatibility, a trait that sometimes is problematic with regards to almond production. Unlike peaches, almonds are self-incompatible and require pollen from a heterologous source as well as an insect vector (bees) for its delivery. The development of self-compatible cultivars will mean that no pollinator trees would be required to set fruit. This will be accomplished by understanding the mechanism of self-incompatibility in almond and the development of transformation technologies that will be used to inactivate the self-incompatible mechanism. Plant transformation involves 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 commercially important variety such as "Nonpareil' with minimal impact on overall characteristics.

Identifying the genes in almond responsible for self-incompatibility: Self-incompatibility (SI) is a widespread mechanism in flowering plants which prevents self-fertilization and promotes out-crossing. The predominant class of SI is the gametophytic SI (GSI) that is controlled by a single locus with multiple codominant alleles referred to as S-alleles. With this type of SI, growth of a pollen tube in the recipient pistil expressing one or any number of S-alleles common to the style leads to the abortion of pollen tube in the stylar tissues. Thus, unless the plant is cross pollinated there is reduced fruit set. The S-alleles encode SRNase proteins that are present in stylar tissue and that are responsible for the inactivation of "self" pollen tubes. Our major objective has been to identify the SRNase proteins and mRNA. Almond has 4 S-alleles, Sa, Sb, Sc and Sd. We have been successful in identifying all four proteins and in determining their N-terminal amino acid sequence. Based upon the amino acid sequence data, we showed last year that the almond S-alleles appear to be unique when compared to the homologs from tobacco and apple. The next task was to identify the mRNA encoding these S-alleles through the analysis of cDNA. We have been successful in identifying cDNA for 3 of the 4 S-alleles; Sb, Sc and Sd. The identity of these cDNAs was confirmed by DNA sequence analysis and this information has been published. The

DNA sequence analysis revealed similarities to other known S-allele genes and the N-terminal sequences we previously determined matched perfectly with the polypeptide predicted from the DNA sequence data. Ultimately, the true functional identity of these clones will only be revealed by antisense experiments. To accomplish this we would need an established transformation system which we are in the process of developing. Currently, we have begun to investigate an alternative approach to confirm the authenticity of our isolated S-allele cDNAs through the development of unique PCR primers based upon our DNA sequence data that will rapidly identify the individual S-alleles. Using DNA from existing almond cultivars whose S-genotypes have been identified we can screen through different primers to identify those that will discriminate the different S-genotypes perfectly. A useful application of these primers would be in the breeding program where they could be used to identify the S-genotype of new varieties from the UCD breeding program, Almond nursery and grower selections. This approach is well underway and we have been able to identify primers that can amplify all 4 alleles including Sa. We are in the process of confirming the PCR products via DNA sequence analysis. If the current data holds true thaen we would have been successful in identifying the DNA sequence of the remaining S-allele, Sa.

Transformation of Nonpareil: Last year we started anew by re-introducing Nonpareil bud wood into culture to maintain clonal authenticity. In the spring of 1998, dormant wood was allowed to push in the lab and the axillary bud tissue was surface sterilized and introduced into culture. Fungal and bacterial contamination was a predominant problem, especially later on in the year when we were using shoot tissue directly from the field. A variety of materials were evaluated for effective sterilization and one of these PPM (Plant Preservative Mixture; Plant Cell Technologies Inc. Washington DC) showed a lot of promise. Typically, our recovery rate with bleach was 41% whereas with PPM we were able to recover 79% of the axillary buds. With the former, most of our losses were due to fungal infestation whereas with the latter (PPM) most of the failures were due to bacterial contamination. Unfortunately, the sterile axillary bud tissue obtained by sterilization with PPM failed to grow *in vitro* whereas the other sterile axillary bud material without PPM pushed in culture and made shoots. Our conclusion was that PPM though effective in sterilizing the plant material was toxic to the regeneration process and therefore not usable.

Once we were successful in obtaining sterile axillary bud material we carried out experiments to optimize axillary bud proliferation and growth in culture. We observed very quickly

that both proliferation and growth was very slow on medium that contains sucrose. Typically, sucrose is a common source of carbohydrate for most plant species. A carbohydrate analysis of Nonpareil shoots using HPLC revealed that sorbitol (the sugar alcohol of glucose) was the major carbohydrate in addition to sucrose. A quanitiative estimation revealed that the ratio of sorbitol to sucrose was about 3:1. When sorbitol was included in the medium, a 60% increase in bud break was observed in comparison to media that contained only sucrose. Fructose, the natural breakdown product of sorbitol in almond, was found to be as effective as sorbitol. Currently, we are investigating various ratios of these two carbohydrates to optimize shoot growth in culture. Next we plan to evaluate the major nitrogen sources in shoots using an amino acid analyzer. This will allow us to identify the appropriate organic nitrogen source that we can include in the media to further optimize shoot growth. Next we will analyze the mineral content using ICP-MS. We hope to duplicate in culture what the almond tissue obtains *in vivo*.

Currently we are investigating the use of different combinations of plant hormones to increase regeneration. The combinations include the plant hormones TDZ, NAA and 2,4D. Combinations of these hormones with GA have not been useful. However, we are begining to identify hormone combinations that promote regenerationa and are testing different concentrations.

Conclusion and future directions: We can conclude from these experiments that we need to keep trying different combinations of the plant hormones to increase regeneration and transformation. In addition we need to evaluate early transformation events in cultures using genes like GFP (Green Fluorescent Protein)and GUS (β-glucuronidase). We plan to continue this effort this year. Our major success has been the cloning of 3 of the 4 S-allele cDNA. Our next task is to identify Sa the remaining S-allele. We have already made very good progress developing a simple PCR approach to identify the S mating type. We need to identify the 4 almond S-RNases using a PCR approach that will be diagnostic of the mating type we hope to accomplish this in the coming year.

Publication:

Ushijima, K., H. Sassa, R. Tao, H. Yamane, A.M. Dandekar, T.M. Gradziel and H. Hirano. 1998. Cloning and characterization of cDNAs encoding the S-RNases in almond (*Prunus dulcis*): primary structural features and sequence diversity of Rosaceous S-RNases. Mol. Gen. Genet. 260: 261-268.

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March 31, 1999

Dear Chris,

Enclosed please find a copy of a final report on our project (Project No. 98-AD-o0) entitled "Genetic engineering of 'Nonpareil' almond".

Thank you for your support.

Sincerely,

Abhaya M. Dandekar
Prof.

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