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Trends of Genetic Study of Forest Tree and Development of Useful Traits for Apple Biotechnology

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It has been estimated that there are more than 250,000 species of flowering higher plants and 750 species of conifers in the world. They are fundamental to the earth's life support system and to human survival. Since the beginning of agriculture, farmers and plant breeders have depended upon genetic variability in plants to develop better traits. Traditional breeders have used natural genetic variation within species. However, rapidly advancing molecular biology and genetic engineering techniques are making progress to enable introduction of useful genes from almost any source. Genetic improvement of forest trees is concerned with the improvement of growth, quality, pest and stress resistance, and the maintenance of a broad genetic base. This is achieved by means of selective breeding, hybridization (cross), clonal propagation, the induction of somaclonal variation, and molecular genetic methods. Other areas include gene conservation and the maintenance of genetic diversity. This paper summarizes recently initiated cultivar development programs involving forest trees and deciduous fruit trees, which were taken, in most part, from the abstracts of Plant and Animal Genome Conference (I to V; 1992-1997). We will also discuss two genetic resources, such as self incompatibility and the MADS-box family gene, which are useful for apple cultivation.

RECENT CULTIVAR DEVELOPMENT PROGRAMS

Genetic Enhancement for Crop Improvement

Plant genetic enhancement is the process of altering the genomic constitution of plants, usually crops, in order to increase their value by improving the yield or the quality of the product from the crop. The molecular analysis of genes has been involved in impor-

tant developmental processes in eukaryotic genomes, of which little is known about their gene products controlling agronomic traits. Molecular genetic markers permit a detailed analysis of plant genomes by allowing the construction of linkage maps, which can provide important bases for studying specific regions of the genome.

Two techniques frequently used in identifying molecular markers defining DNA target sequences are DNA hybridization and polymerase chain reaction (PCR). Restriction fragment length polymorphism (RFLP) based markers have been widely used in the construction of genetic maps and in the identification of mono and polygenic loci controlling agronomic traits. However, the main drawback of RFLP analysis is that it involves many steps that are quite difficult to automate. DNA amplification products obtained from PCR analysis using random primers (RAPD) have been proposed as an alternative method in targeting DNA sequences for genetic characterization and mapping. Genetic maps incorporating RAPD-based markers have been reported in several important crop species. Near isogenic lines have been used to identify RAPD-based markers linked to resistance genes for pathogens. In the absence of near isogenic lines, bulk segregant analysis is useful for the identification of DNA sequences linked to a target region. Bulk segregant analysis is a process by which DNA from selected individuals in segregating progeny homozygous for specific alternate alleles is pooled and then examined for molecular marker differences. RAPD-based markers and bulk segregant analysis were used in apples to identify DNA markers linked to scab resistance (Yang *et al.*, 1997) and the powdery-mildew resistance gene (Markussen *et al.*, 1995).

Important Wood Properties

Genetic mapping with molecular markers has become a valuable tool for identification of important traits in tree breeding. Quantitative trait loci (QTL)

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mapping of wood qualities is particularly valuable because marker aided selection of economically important wood properties would enable identification of superior tree at a younger age. There are RAPD markers currently developing for mapping QTLs of wood density in the genera *Pinus* and for providing a frame work to test for QTLs of important traits with major effects on stem growth, form, leaf phenotype, wood quality, disease resistance, and organogenesis *in vitro* in the genera *Eucalyptus* and *Populus*. Among these, the most important wood property is specific gravity (woody density). It affects all products that can be made from wood, from the strength of timber to the ease of machining wood for fur-

niture to the quality of pulp and paper. Other traits of importance are fiber length, diameter, wall thickness, angle, and chemical composition. In hardwoods, most of the work has been done with conifers, eucalyptus, poplars, and white birch, which have economic value.

In forest tree improvement, the greatest limitation is long generation time. To accelerate the breeding cycle, two complementary approaches are practiced. One is to shorten the period preceding flower induction or bypass it altogether. The other is to shorten the period needed to test and evaluate the quality of trees. Early testing methods have been effective in the elimination of undesirable trees at an ear-

Table 1. Survey of the genetic research programs in major forest trees.

Species	Methods	Objective	Institute
<i>Pinus</i> (Loblolly Pine, Slash Pine, Monterey Pine)	Identification of QTL	For Influencing Wood Specific Gravity	North Carolina State Univ., USA
	Molecular & Cytological Analysis of Repeated DNA Sequences	Identifying the Polymorphic Regions	USDA Forest Service, USA
	Codominant PCR-Based Markers Using cDNA Clones	Application in Genetics	USDA Forest Service, USA
	Developing RAPD Markers for Mapping QTLs	Wood Density Analysis	Forest Research Inst., NewZealand
	Genome Mapping	Marker Assisted Progeny Selection	NC State Univ., USA
	Mapping Genes Expressed during Wood Formation	Methods of Mapping cDNAs for Identifying & Mapping Polymorphism	NC State Univ., USA
<i>Eucalyptus</i> (<i>E. grandis</i> , <i>E. urophylla</i>)	Linkage Map & QTL Analysis	For Traits Assessed in Seedlings	CSIRO & ForBio Research, Australia
<i>Betula Pendula</i> (Birch)	RAPD Markers	Parentage Analysis	Finnish Forest Research Institute, Finland
	Microsatellite Markers	Developing the Codominant & Microsatellite Markers	Kuopio Univ., Finland
<i>Populus</i>	AFLP Markers	Fingerprinting Genetic Marker	Biotechnology & Food Research, Finland
	Genome Mapping	Resistance to Rust Races	INRA, France

*From Abstract of Plant and Animal Genome Conference I-V.

ly age. There is a greater potential for DNA markers to improve genetic analysis and to accelerate breeding in forest trees. Marker aided selection would be particularly useful to shorten the selection phase. Many researchers are currently involved in doing this project (Table 1).

Important Properties of Orchard

The fruit, which has high nutritional value and also provides beauty to the landscape, plays a role in contributing a positive influence on the world environment. Citrus fruits are the world's number one fruit crop in production (77 million tons in 1991) followed by grapes, bananas, and apples (57, 47, and 39 million tons, respectively) deciduous fruit plants. In Korea, the apple is the most important cultivated fruit species. Unlike annuals, perennial fruit plants cannot be managed in a short-term rotation plan. These perennial plants reside in one location for 25 to 100 years. Numerous features of deciduous fruit plants

distinguish them from annuals, most of which are related to differences in vegetative and reproductive cycles of perennials and annuals. All life forms begin development at a juvenile stage and grow into an adult stage when reproduction may occur. The process of passing from juvenile to adult stage takes weeks for annual plants, while it takes several years for deciduous fruit trees. A curious feature of the reproductive stage in deciduous trees is that their vegetative and reproductive events occur over a two year cycle (alternative bearing), whereas in annual plants, they take place in one year. In deciduous fruit trees, cultivar development programs have showed little effect because of impediments associated with reproductive biology and the lack of efficient selection strategies. Genome characterization and mapping projects have provided information that will be of practical value to deciduous fruit breeding programs. There are DNA-markers currently developing that can be utilized for indirect selection of desirable hybrids for resistance to viruses and fungi, dwarfing, and for a

Table 2. Survey of the genetic research programs in major orchards.

Species	Methods	Objective	Institute
<i>Prunus</i> (Peach, Almond, Cherry, Plum)	Genetic Linkage & Chromosome Mapping & QTLs	RFLP, RAPD & Morphological Marker	For Genes Controlling Canopy Shape, Fruit flesh Color
			Resistance to Pathogens & Pest
<i>Citrus</i>	Genome Mapping & QTLs	RFLP, RAPD Marker	Resistance to Virus, Nematode, Phytophthora, Iron chlorosis & Tolerance of Salinity, Cold Temperature & Dwarfing
Apple	Genome Mapping & Genetic Linkage Map	RFLP, RAPD Marker	Resistance to Scab, Scald, Powdery-mildew & For Genes Controlling Fruit Color & Columnar habit
	Genome Mapping	Molecular aided breeding	For Genes Controlling Tree habit & Fruit quality
			Seven European Countries

*From Abstract of Plant and Animal Genome Conference I-V.

gene that influences fruit quality traits such as acid accumulation, sugar content, columnar habit in the genera *Citrus* and *Prunus*, and apple species (Table 2).

Methods of Functional Analysis of Wood Genome Study

There is much interest in applying genetic engineering techniques to forest trees, but there is little basic information to guide decisions about which genes to introduce in order to alter of tree phenotypes. Since forest trees, which have long generation intervals and possess an extremely large genome and low transformation efficiency are typically outbred, they are not amenable to the forward genetic (mutant screen/positional cloning) analysis that has been so productive in *Arabidopsis* and other model systems. An adaptation of a reverse genetic approach would be to use antisense constructs to create single mutations in transgenic plants. The principle of the technique is based on the observation that nucleic acid hybridization between mRNA and its complementary RNA sequence prevents the translation of the mRNA. Lignin is a the most abundant organic compound in the biosphere. During plant development, lignin is deposited in secondary cell walls and provides rigidity and structural support to the cell walls, enabling solute conductance in the vascular system. Despite its important biological role, it needs to be removed during the paper making process. The O-methyl transferase is a potential target enzyme for reducing the amount or modifying the composition of lignin in plants. Successful examples of the antisense RNA approach are the suppression of the transcripts of the endogenous O-methyl transferase gene in the stem of the tobacco (Dwivedi *et al.*, 1994) and poplar (Doorselaere *et al.*, 1995). This technique could also be applied to the genes involved in signal transduction pathways and transcription factor genes such as *Myb* homologues which are expressed in developing wood (differentiating xylem), and MADS-box genes which play a key role in flower development, because these genes affect important processes including the expression of numerous downstream genes.

In vitro propagation techniques are essential to make genetic engineering techniques practical. Since most of the transformation is done in tissue culture with single cells, techniques need to be available to regenerate whole plants. Unfortunately most forest trees are rather recalcitrant in this respect, so this obstacle hinders the analysis of gene function. An alt-

ernative approach is to utilize other plant systems such as the tobacco, which is easily transformed. For these reasons, ectopic expression approach is routinely used for characterization of genes which are isolated from wood plants.

USEFUL TRAITS FOR APPLE BIOTECHNOLOGY

Self Incompatibility and Self Pollination

In most fruit species, pollination is accomplished by insects such as bees, whereas others, such as pecan and walnut, are wind pollinated. Strong winds and rain can preclude effective pollen transfer. Temperatures below 18°C can also be detrimental as insects fail to work under such conditions unless given time for adaptation. The air pollution resulting from burning various fuels leads to the reduction of the number of insects and eventually hinders fertilization. The apple is a gametophytic self-incompatible (SI) plant, since they need pollen donor cultivars for pollination. The economic value of the apple tree relies on its fruits. Therefore, the expression of SI genes in apples is often disadvantageous, since two different cultivars must be grown next to each other. Usually 10% of the total fruit trees are pollinator trees, serving mainly as a pollen donor and generally yielding fruits of lower quality. Understanding of the self-incompatibility system in the apple species may lead to improvement of the yield and enhancement of apple

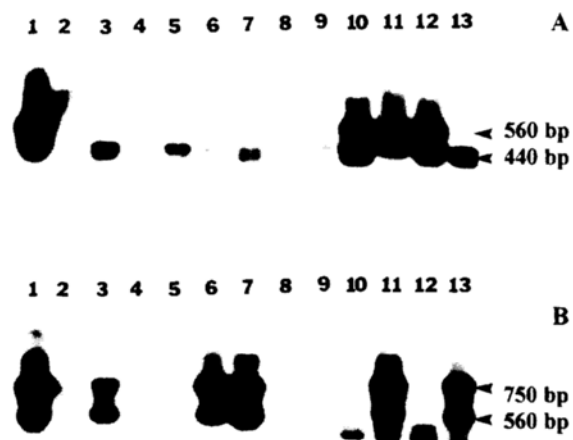


Fig. 1. DNA blot analysis of the PCR amplified SI genes with SIF1 (A) and SIF2 (B). Fuji (1), Molloy's Delicious (2), Rall's Janet (3), Golden (4), Tsugaru (5), *M. Sieboldii* (6), Mutsu (7), *M. spp.* VanEseltine (8), Jonagold (9), *M. Baccata* (10), Hokuto (11), Chukwang (12) and *M. prunifolia* (13).

cultivars. For these reason, we have identified and characterized SI genes in the Fuji apple (Sung *et al.*, 1996). Currently, we are trying to generate a self-pollination cultivar using the anti-sense RNA approach. Another utilization of the apple SI genes is, because SI genes determine the barrier between cultivars, to establish the relationship among different cultivars. We have tried to explain the diversity of S alleles between different cultivars, and this approach can be used for studying the relationship between pollen donor and recipient (Fig. 1).

Flowering and Thinning

Deciduous fruit trees typically produce more flowers than are needed for a fruit crop of desirable size and quality. One mature apple or peach tree may produce 50,000 flowers from which about 2,500 fruits are needed for an economic crop. If more than 2,500 fruits per tree are formed and allowed to remain, the resulting fruit will be small and have poor quality, variable maturity, and finally, flower bud production for the next year will be reduced. For these reasons, the flower or fruit population must be reduced (thinned) early in the growing season. Some fruit species such as apples and peaches are thinned by hand, with a labor intensive operation, or thinned by chemical sprays. Chemical fruit thinning can be accomplished from the time of flowering until to about 30 days later. Great variability in chemical fruit thinning success occurs mainly due to weather conditions which are apt to be less than optimum at thinning time, resulting in over or under thinning. Also, fruit thinning chemicals used well after flowering cause abscission of young fruit by interfering with normal fruit metabolism. Some thinning chemicals act by interrupting the transport of chemicals into and out of the fruit. For most fruit species, including the apple, chemical fruit thinners have not been reliable enough to completely eliminate the need for hand thinning. Therefore, there is need for fruit tree varieties that will regularly produce enough flowers for a commercial crop without requiring fruit thinning, while at the same time forming just enough floral buds for the next year's crop.

There are various horticultural strategies to control the numbers of flowers and fruits and to offset alternate bearing. The best strategy is to control the fruit population by fruit thinning. When fruit thinning is not possible because of lack of an acceptable procedure, other horticultural measures should be thought of. Such measures include judicious pruning

of vegetative growth, careful pest control, and careful timing for fertilizer. More variable controls become possible by limiting cross-pollination and the use of varieties that naturally bear fewer flowers each year. Another possible approach is to investigate flower and fruit development based on molecular biology. For example, using the differential display (DD)-PCR and expressed sequence tag (ESTs) techniques, we can identify the cDNA clones that are specifically expressed at the early stage fruit development. The clones can be used for engineering the apple to bear the optimum number of fruits.

Flower Development and MADS-Box Family Genes in the Apple

In most angiosperms, flowering is induced in response to environmental conditions such as day length and temperature. The development of each of the flower organ in *Arabidopsis* has been explained by the combined action of the genes responsible for flower meristem identity according to the ABC model (Weigel and Meyerowitz, 1994), and some of these genes are also involved in meristem determination. Several genetic pathways interact to control the onset of flowering in response to environmental signals. In *Arabidopsis*, a long day flowering plant, it could be achieved, presumably, through the activation of both the floral meristem identity gene (*APETALA1*; Bowman *et al.*, 1993) and flowering time gene (*CONSTANS*; Putteraill *et al.*, 1995). Most known floral homeotic genes belong to the MADS-box family, and their products act in combination to specify floral organ identity by an unknown mechanism. MADS-box proteins are involved in a diverse range of biological activities. A large number of MADS-box genes have been identified from various plant species. These genes are expressed in a number of tissues but the most extensively characterized are those which dictate floral organ identity such as those found in *Arabidopsis* and *Antirrhinum majus*. Floral meristem genes, *API* and *SQUAMOSA* contain the MADS-box motif, meaning other MADS-box genes may have similar functions in other plants. Previously, we have isolated several MADS-box genes from rice, and their functions were analyzed by ectopic expression in transgenic tobacco. We have shown earlier that one of the MADS box genes, *OsMADS1*, can induce an early flowering and semi-dwarf phenotype when the gene was expressed by the 35S promoter (An, 1994; Chung *et al.*, 1994).

In contrast to the wealthy amount of information a-

bout the regulatory genes controlling floral meristem and organ development in annual plants, very limited information is available concerning these genes in woody plants. There has been only one report on the molecular study of the floral development in the fruit trees of the *Rosaceae* family (Sung and An, 1997).

Previously, we isolated four MADS-box genes (*MdMADS1-4*) in the Fuji apple. To understand the role of the apple MADS-box genes, RNA blot analysis was performed. The transcript levels of these apple MADS-box genes were highest at the early stages of flower and fruit development. We ectopically expressed the *MdMADS* genes by placing them under the control of the 35S promoter and transforming the chimeric genes in tobacco. From this study we found that some of these MADS-box gene can be induce an early flowering.

CONCLUSION

Forest trees have long generation times and are highly heterogeneous, so few extended pedigrees are available. Because of these limitations, there is a greater potential for DNA-markers to improve genetic analysis and to accelerate breeding in forest trees. In deciduous fruit plants, the most important traits are fruit qualities such as acidity, sugar content, fruit size, and total yield. As these are quantitative traits that appear to be controlled by a number of loci, it is difficult to make progress in the quick improvement of fruit quality, requiring the elucidation of the position and function of QTLs affecting these traits. Although QTL analysis is important for genetic enhancement, it is time consuming work. Also, for many years until now, DNA-markers have not been applied to practical tree improvement because of technical and theoretical limitations. High levels of heterozygosity and linkage equilibrium of markers in populations were considered to be serious limitations. Now, it is carefully suggested that molecular breeding should be primarily based on the cloning and characterization of useful agronomic traits for direct application to forest tree plants. For example, a monogenic trait that specifically contributes to flower and fruit development is useful for control of thinning. Biotechnology is likely to give valuable genetic improvement of tree

species and the adopted strategy based upon biotechnology provides a model system in similar studies for other fruit and woody species.

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