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TREE BIOTECHNOLOGY: RECENT UPDATES ON GENETIC TRANSFORMATION OF CONIFERS

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Advances in somatic embryogenesis using apical shoot buds of mature conifers have brought mass clonal propagation of the top commercial trees closer to reality, and efficient gene transfer systems have been developed for a number of conifers. Embryogenic tissue derived from the cloning of mature pines serves as the best starting material for genetic transformation studies in conifers. The first transgenic trees were produced in an Indian pine *P. roxburghii* following biolistic gene transfer using embryogenic tissue of mature trees, followed by the *Agrobacterium*-mediated genetic transformation of embryogenic tissue of mature Himalayan blue pine (*P. wallichiana*). This is the major breakthrough in forest biotechnology and might help in solving the current problems of transformation of recalcitrant pines and has many potential applications in commercial forestry. In another development, the homeobox transcription factor *WUSCHEL* (*WUS)* has been shown to cause dedifferentiation when expressed on somatic cells derived from apical meristematic tissue followed by a production of new cells that can lead to somatic embryogenesis in *P. roxburghii* has many practical applications in commercial forestry. Therefore, there is a possibility of utilizing this *WUS* gene in the cloning of mature conifers.

Keywords : Cloning; Conifers; Genetic transformation; Indian pines; Mature trees.

Introduction

Trees are an integral part of human life, and a vital component of biodiversity. Forest trees in particular are renewable sources of food, fodder, fuel wood, timber and other valuable non-timber products. The global need for wood is not expected to decrease in the near future; in fact the demand for several wood products (especially paper, pulp and biofuel energy) is more likely to increase[1]. Due to rapid growth of population and the human desire to progress, there has been a tremendous reduction in forest cover from earth's surface. To maintain and sustain forest vegetation, conventional approaches have been exploited in the past for propagation and improvement. However, such efforts are confronted with several inherent bottlenecks. Against the background of limitations of long juvenile phases and life span, development of plant regeneration protocols and genetic engineering of tree species are gaining importance. Genetic engineering assumes additional significance, because of the possibility of introducing a desired gene in a single step for precision breeding of forest trees[1-10]. To lower the pressure on existing forests, mainly rain and conifer forests, a global effort is needed, to include trees in the modern era of plant breeding. Plantation forestry, with optimized and increased forest productivity, is likely to become the major source for wood products in the future and so accelerated tree improvement biotechnological programs are key elements for the successful reforestation and management of future commercial forests[1]. Currently, most tree-breeding programs are based on the management of genetic resources, including selection of superior clones from existing forests, the conservation of genetic variability, partially controlled propagation and classical breeding for desired traits[1]. Hitherto, breeding for wood traits has been

hampered by the cost of traditional assays, the need to wait until trees are nearly mature to be evaluated, high heterozygosity and autoincompatibility[1]. In practice, significant progress in the breeding of many forest-tree species is limited because of the long lag time between seed germination and flowering, and because most of the relevant forest-tree traits can be assessed only when the tree reaches maturity[10-27]. Limited knowledge of the genetic maps of most forest trees is yet another limitation; there is a lack of genes for hybridization and expression of new traits, with those that are known being mainly for novel genotypes and tolerance to biotic and abiotic stress. Moreover, the identification of suitable parents and the technical difficulties involved in their controlled mating add to the limitations of forest-tree breeding. Even though several superior hybrid trees with accelerated growth, altered form and environmental adaptations have been obtained through classical breeding, their maintenance is problematical because of the high heterozygosity of forest trees which are mainly propagated by seed. Finally, the size of forest trees and the area required for field trials create considerable difficulties in assessing their performance. Plant transformation techniques and gene isolation, and characterization are no longer serious obstacles, and so forest trees should be a major target for genetic engineering and molecular breeding. The potential of biotechnology for overcoming many of these limitations and for accelerating forest tree-breeding programs can be realized at several levels, clonal propagation of superior genotypes using tissue culture techniques and direct rapid introduction of specific traits *via* genetic engineering of forest-tree species. Plant genetic transformation, the controlled introduction and expression of foreign genes in plants-has become a common technique both for basic research and for the introduction of novel traits into commercially important species [1-14]. Different tools are now available to transform plants genetically, and the most commonly used-*Agrobacterium* and particle bombardment have been extensively used [1-27]. This review focus on the advances that have been made in the genetic transformation of conifers and the possible applications of transgenic trees in modern forestry.

Background - Somatic embryogenesis in many conifers throughout the world using vegetative shoot apices or secondary needles which provides the best opportunities to produce transgenic plants in a number of species that will lead to their application in commercial forestry [1-27]. The first transgenic trees produced by using embryogenic tissue derived from the vegetative shoot apices of mature trees were reported in an Indian pine, *Pinus roxburghii*[2,3]. This is the major breakthrough in forest biotechnology, and certainly solves the current problems of tree breeding. With the various gene transfer methods currently available, simple placement or transfer of DNA into a plant cell is no longer a limiting factor. However, both the mechanisms for DNA transfer to a plant cell and targeting of the DNA to a complex tissue or organ competent for regeneration is another major issue to be considered for effective and successful transformation. Now-a-days there are many genes available for use in conifer transformation experiments. However, most of those have been used as reporter genes for establishing a model transformation system, and very few have been used for novel phenotypes or for tolerance to various stresses. A model transformation system is very much needed before transfer of an economical trait gene into conifer tree species can be accomplished. However, many cultivars of those transgenic tree lines are now in field trials [1-27].

Methods of genetic transformation and problems-There are two powerful methods of gene transfer, currently available. One is co-culture method using *Agrobacterium* and another one is particle bombardment method. Among these methods, biolistic gene transfer method has been used the most widely for generating transgenic conifer trees, and the delivery of transgene into embryogenic tissues by particle bombardment remains the principle direct DNA transfer technique in plant biotechnology. In this method there is no dependence on bacteria, so the limitations inherent in organisms such as *A. tumefaciens* do no apply. The absence of biological constraints, at least until DNA has entered the plant cell, means that particle bombardment is a versatile and effective transformation method, not limited by cell type, species or genotype. There are no intrinsic vector requirements so transgenes of any size and arrangement can be introduced and multiple gene co-transformations are straightforward. Plant transformation method by *A. tumefaciens*, soil pathogenic bacterium, has become the most used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants. *A. tumefaciens* has the exceptional ability to transfer a particular DNA segment (T-DNA) of the tumor-inducing (Ti) plasmid into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed causing the crown gall disease. Ti-plasmids are classified according to the opines, which are produced and excreted by the tumors they induce. The process of T-DNA transfer is mediated by the cooperative action of proteins encoded by genes determined in the Ti plasmid virulence region (*vir* genes) and in the bacterial chromosome. The

Ti-plasmid also contains the genes for opine catabolism produced by the crown gall cells, and regions for conjugative transfer and for its own integrity and stability. Although, the gene transfer mechanisms remain largely unknown, great progress has been obtained in the implementation of transformation protocols for many plant species including conifers. Particularly important is the extension of this single-cell transformation methodology to recalcitrant pines. This advance has biological and practical implications. As the ability to transform conifer species is becoming a reality, researchers and breeders should be aware of the problems. During its perennial life cycle, a forest tree must adapt to seasonal climatic changes and to a wide range of pests and abiotic stresses. Consequently, tree populations exhibit high diversity, reflected in their many ecotypes; theres is , therefore, a need to transform different ecotypes for stable expression of the transgenes through cycles of environmental changes. In addition, the long life cycle of forest trees calls for stability of the transgenes over several years. As the common constitutive promoters (Cauliflower mosaic virus 35S and mannopine synthesa (MAS) are silenced in many annual transgenic plants, unique constructs with more suitable promoters, preferably of tree origin, are likely to be required for the long-term expression of foreign genes in forest trees.

Targets for forest-tree engineering- There are many applications of gene transfer technology in commercial forestry programmes which can solve the current problems of breeding, such as lignin modification, phytoremediation, altering tree form, quality and performance, herbicide resistance, insect resistance, abiotic stress tolerance, flowering control, wood modification etc. Among all these applications, modifying lignin content in forest trees has been advantageous. Lignins represent a serious problem to efficient pulp and paper production, for which they must be removed in an energy-consuming process that involves the use of polluting chemicals. Engineering for reduced lignin content was first reported in downregulated phenylalanine-ammonia-lyase (PAL)-transgenic tobacco plants. However, because PAL is a key enzyme in the shikimate and phenyl-propanoid pathways, the plants also exhibited pleiotropic effects (stunted growth, and altered flower morphology and pigmentation). Several enzymes have been identified from the lignin-biosynthetic pathway. Although it is clear that lignin content and composition can be modified in genetically engineered trees, many of the enzymes and reactions in the lignin-biosynthetic pathway still need to be characterized. Phytoremediation is another possible target for transgenic conifers. The use of plants to clean up environmental pollution was suggested by either stabilizing the pollutant in the soil or eliminating it from the polluted soil. The other aspect of transgenic trees is the altering tree form, quality and performance in which wood quality is needed to be improved for the commercial purposes. Wood quality is defined in terms of density, absence of knots and uniformity. Genetic manipulations of flowering genes are mainly aimed to shorten flowering and generation time. Another problem specific to tree species, compared to conventional agricultural crops, is the necessity for long term stability of the transgene over several vegetative periods. Increase in knowledge about the control of flower development in trees opens up strategies to reduce or prevent the danger of vertical gene transfer to the wild tree species via genetic engineering of sterility. These genes play important roles in both the formation of the flower meristem and the determination of floral organ identity. They also reported that one of these genes, M8p-1 was expressed stronger in the male strobilus and that its transcriptional activity was increased as the male strobilus developed. Herbicide-resistant transgenic crops are considered to be one of the genetic engineerings major successes. Increased herbicide tolerance was also achieved in transgenic plants by substituting the 35S promoter for the MAS promoter. The overuse of herbicides is not as harmful in transgenic forest trees but can lead to the accelerated selection of herbicide-resistant weeds and to environmental pollution. Genetic engineering for insect control has been achieved in several annual plants, using either the *Bt* toxin (from *Bacillus thuringiensis*) or insect-digestive-system-inhibitor genes.

Limitations - Tree improvement is a very costly and long-standing process, in terms of time, expenditure and availability of technology. Therefore, in order to achieve this goal, basic infrastructure like a good laboratory facilities, with well developed molecular biology technology are needed for the success of genetic transformation[1-3]. This could be possible only with a long term funding from the government or from the public sector. At this time still it is very difficult to convince the corporate or government for getting funding for this kind of work particularly genetic transformation of plants including conifers. Unfortunately, there is no guarantee that a transformation plant cell type will prove regenerable since current tissue protocols at least in a few conifer species used in the transformation studies are very poor and found not to be reliable and reproducible. This is the main drawback and hinders the success of genetic transformation. Tissue culture is an important prerequisite

for the successful genetic transformation studies. There is a growing fear in the public that there will be an imbalance in the ecological niche, and the arrival of new disease to the living organisms including human beings on this earth. Funding agencies are not any more interested in such type of projects. Due to the lack of funding for many projects on genetic transformation, the work progress have been suffered and ended up with baseless results. These results can not be utilized for the commercialization of genetic transformation protocol but ended up with basic results for just scientific research. Trees live longer than agricultural crops, which means that changes in their metabolism may occur many years after they are planted. At the same time, trees are also different from crops in that they are largely undomesticated and scientists' knowledge about forest ecosystems is poor. This implies that the ecological and other potential risks associated with GM trees are far greater than in the case of crops. Long-term field studies should be designed to examine not only novel genes stability and transgenic behavior but also tree-crop-induced fluxes in soil nutrient status and soil water availability [1-4].

Conclusion

Forest tree improvement has now been slow and arduous process by virtue of the large size and long generation times of trees. Biotechnology has now provided tools, however, that allow us to select and engineer superior trees with much the same speed and efficiency that can be applied to other organism. It is envisaged that a combination of biotechnological tools and classical breeding techniques will provide the most benefit to the improvement of forest tree species. The genes can then be introduced through transformation methods into selected tree species and the transgenic trees cloned and propagated in tissue culture. It is concluded that particle bombardment and *Agrobacterium*-mediated genetic transformation are likely to continue to play an important role in plant biology and forest biotechnology for many years into the future. When these goals are realized, future forest trees will be more tolerant to abiotic and biotic stresses, express genes for accelerated growth rate, and have a modified wood structure. Such trees will bring forestry into a new era of productivity and quality. However, the impact that such tree may have on ecosystems when planted on the plantation scale needs much additional study.

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